

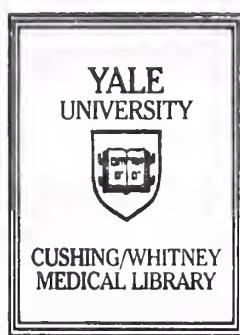
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SPONTANEOUS DERMATITIS IN  
GAMMA/DELTA T CELL DEFICIENT MICE:  
THE ROLE OF IgE, GENETICS, AND  
DENDRITIC EPIDERMAL T CELLS

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YALE UNIVERSITY

2003



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SPONTANEOUS DERMATITIS IN  $\gamma\delta$  T CELL-DEFICIENT MICE:  
THE ROLE OF IgE, GENETICS, AND DENDRITIC EPIDERMAL T CELLS

A Thesis Submitted to the  
Yale University School of Medicine  
In Partial Fulfillment of the Requirements for the  
Degree of Doctor of Medicine

by

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Dedicating an extra year in medical school to research in the Yale Department of Dermatology is one of the most fortunate and blessed decisions I have made in my medical school career. Little did I know that it would bring me not only the privilege to work with some of the most intelligent, dedicated, and inspiring individuals I have ever met in medicine and academics, but also the unexpected gift of precious relationships and friendships with loving, caring, and selfless individuals I have been fortunate enough to meet in this Department and around the lab.

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## SPONTANEOUS DERMATITIS IN $\gamma\delta$ T CELL-DEFICIENT MICE: THE ROLE OF IgE, GENETICS, AND DENDRITIC EPIDERMAL T CELLS

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Atopic dermatitis (AD) is a chronic inflammatory skin disease characterized by intense pruritus and chronically relapsing eczematous skin lesions, often associated with elevated serum IgE levels. It has previously been reported that non-obese diabetic (NOD)  $\gamma\delta$  T cell-deficient ( $\delta/-$ ) mice, but not C57BL/6 (B6) or (NOD X B6)F<sub>1</sub>. $\delta/-$  mice, develop an  $\alpha\beta$  T cell-dependent, environmentally-influenced, localized spontaneous dermatitis (SpD) resembling human AD. Reconstitution studies further demonstrated that the SpD is down-regulated by V $\gamma$ 5+ dendritic epidermal T cells (DETC). We analyzed whether IgE levels correlated with genotype and/or the SpD phenotype. Total serum IgE levels (ELISA) were determined in B6 wild-type (w.t.) and B6. $\delta/-$  mice, NOD w.t. and NOD. $\delta/-$  mice, and (F<sub>1</sub> X NOD). $\delta/-$  (BC) mice, raised in ventilated and non-ventilated housing conditions. Higher IgE levels were found in NOD w.t. and NOD. $\delta/-$  mice compared to B6 w.t. and B6. $\delta/-$  mice. Total serum IgE levels did not correlate with presence of SpD, nor with the environment in which they were housed. These data suggest that IgE antibodies do not play a primary role in the pathogenesis of SpD. Genome-wide microsatellite mapping studies in other AD mouse models have identified major susceptibility intervals to chromosome 9 in NC/Nga mice and to chromosome 14 in NOA mice. Using primers distinguishing NOD and B6 polymorphisms in the centers of these intervals, DNA from BC. $\delta/-$  mice with SpD was analyzed by PCR. For both intervals, the NOD/NOD *vs.* NOD/B6 ratios were 1.0, indicating that the interval(s) controlling susceptibility to SpD in NOD. $\delta/-$  mice are distinct from those found in other mouse models of AD. Finally, in search of ultimately defining the ligand(s) for the DETC-specific V $\gamma$ 5/V $\delta$ 1 T-cell receptor (TCR), a third goal was to develop a bioassay in a heterologous cell type, a rat basophil leukemia cell line, RBL-2H3. A RBL-2H3 cell line expressing the V $\gamma$ 5/V $\delta$ 1 TCR was developed, and preliminary studies performed in which pre-loaded  $^3$ H serotonin was measured for release from V $\gamma$ 5/V $\delta$ 1 transfectants upon stimulation. The RBL bioassay is a first step towards defining the ligand(s) for the DETC-specific V $\gamma$ 5/V $\delta$ 1 TCR, which will help to elucidate the physiological role of this intriguing subset of  $\gamma\delta$  T cells.



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## LIST OF ABBREVIATIONS

A	Adenine base
AD	Atopic dermatitis
APC	Antigen presenting cell
$\beta_2$ M	$\beta_2$ -microglobulin
B6	C57BL/6
BC	Backcross
bp	Base pairs
BSA	Bovine serum albumin
C	Cytosine base
C region	Constant region gene segment of TCR
cDNA	complementary DNA
CDR	Complementarity-determining region
cM	centiMorgan
CMEM	Complete MEM
CNVC	Conventional non-ventilated cages
ConA	Concanavalin A
Csk	Cytoplasmic tyrosine kinase
D region	Diversity region gene segment of TCR
DC	Dendritic cell
ddH <sub>2</sub> O	Double-distilled water
DETC	Dendritic epidermal T cell
DMBA	dimethylbenz( <i>a</i> )anthracene
DMEM	Dulbecco's Modified Eagle's Medium
DN	Double-negative
DNFB	1-fluoro-2,4-dinitrobenzene
DTT	2,3-dihydroxy-1,4-dithiobutane
EAD	“Extrinsic” atopic dermatitis
EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme linked immunosorbant assay



FACS	Fluorescence Activated Cell Sorting
FCS	Fetal calf serum
FGFVII	Fibroblast growth factor VII
FITC-	Fluorescein conjugated
G	Guanine base
GM-CSF	Granulocyte-macrophage colony stimulating factor
GVHD	Graft-versus-host disease
Hsp	Heat shock protein
IAD	“Intrinsic” atopic dermatitis
IEC	Interface epidermal cell
IEL	Intraepithelial lymphocyte
Ig	Immunoglobulin
IL	Interleukin
IFN	Interferon
ITAM	Immunoreceptor tyrosine-based activation motif
IVC	Individually ventilated cages
J region	Joining region gene segment of TCR
kD	Kilodalton
KGF	Keratinocyte growth factor
LB	Luria broth agar
MCA	Methylcholanthrene
MCP	Monocyte chemoattractant protein
MCC	Mast cell chymase
MEM	Minimum Essential Medium
MHC	Major Histocompatibility Complex
MoAb	Monoclonal antibody
mRNA	Messenger RNA
N region	Non-germline encoded nucleotide region of TCR
NOA	Naruto Research Institute Otsuka Atrichia
NK	Natural killer



NOD	Non-obese diabetic
NP-40	Nonident P-40
PAF	Platelet-activating factor
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE-	Phycoerythrin-conjugated
PLC	Phospholipase C
PMA	Phorbol myristate acetate
PTD	Post-transfection day
QTL	Quantitative trait locus
RAG	Recombinase Activating Gene
RBL	Rat basophil leukemia cells
rpm	Rotations per minute
RT	Reverse transcriptase
SD	Standard deviation
SEM	Standard error of the mean
SpD	Spontaneous dermatitis
SPF	Specific pathogen-free
SSLP	Simple sequence length polymorphism
T	Thymine base
TAE	0.04 M Tris-acetate, 0.001 M EDTA
TCR	T cell receptor
TdT	Terminal deoxyribonucleotidyl transferase
Tg	Transgenic
TGF	Transforming growth factor
Thy-1+ DEC	Thy-1+ dendritic epidermal cell
TMB	Tetramethylbenzidine
TNF	Tumor necrosis factor
TPA	12- <i>O</i> -tetradecanoylphorbol
U	Units
V region	Variable region gene segment of TCR



## I. INTRODUCTION

### A. BACKGROUND OF $\gamma\delta$ T CELLS

T cells are critical effectors of the vertebrate adaptive immune system. In most mammals the vast majority of recirculating T cells in the blood and peripheral lymphoid tissues (>90%) express an antigen-specific T-cell receptor (TCR), which includes a disulfide-linked heterodimeric glycoprotein composed of a 42-45 kilodalton (kD)  $\alpha$  chain and a 35-42 kD  $\beta$  chain; each chain contains a variable and constant domain, a transmembrane region, and a relatively short cytoplasmic tail [1]. The  $\alpha$  and  $\beta$  chain together serve to recognize specific antigens. Conventionally viewed as thymus-derived cells that recirculate through lymph nodes and spleen via blood and lymph, these so-called  $\alpha\beta$  T cells enter tissues following antigen-specific activation and mediate reactions designed to control infection by pathogens foreign to the host [2]. The  $\alpha\beta$  T cells are associated with either the CD4 or CD8 co-receptor cell-surface protein. CD4 T cells recognize only peptides presented by self-Major Histocompatibility Complex (MHC) class II molecules on the surface of target cells and differentiate according to their cytokine pattern into CD4 T<sub>H</sub>1 and T<sub>H</sub>2 effector cells that activate macrophages and B-cell responses to antigen, respectively. CD8 T cells, on the other hand, recognize peptides bound only to MHC class I molecules, and differentiate into cytotoxic CD8 T cells [2]. The TCR as a whole is composed of the ligand-binding heterodimer closely associated with four additional, nonpolymorphic polypeptide chains [3]. These nonpolymorphic transmembrane polypeptides, namely CD3 $\epsilon$ , CD3 $\gamma$ , CD3 $\delta$ , and TCR $\zeta$  chains [4], are collectively known as the CD3 complex, which plays a critical role in



receptor assembly and cell-surface expression [5]. In addition, the CD3 complex is the key through which signals are transduced from the TCR to the cell upon TCR activation and cross-linking by antigen [5]. Antigen recognition and functional activities of  $\alpha\beta$  T cells are understood in great depth, and therefore, have often been generalized to all T cells. It is only in the recent past that we have begun to understand a distinct set of T cells, comprising a minority of recirculating T cells (<10%), known as  $\gamma\delta$  T cells [6, 7].

The study of  $\gamma\delta$  T cells is relatively recent and stems from the discovery of their rearranged genes, rather than from any knowledge of their biological function [8]. The existence of  $\gamma\delta$  T cells was not suspected when the TCR  $\gamma$  gene was first cloned independently from the genes for TCR  $\alpha$  and  $\beta$  in early 1984. Several years passed before cells expressing the  $\gamma$  gene product were discovered, which were found to express this  $\gamma$  chain as part of a CD3-associated heterodimer in conjunction with a second unidentified chain [9-15]. Within the next three years, the other TCR subunit of this unique heterodimer was discovered and designated TCR  $\delta$ . The gene encoding it was identified based upon gene rearrangement and subtractive hybridization [9, 16, 17], with its location found to be within the TCR  $\alpha$  locus [8, 18]. Further studies showed that cells expressing  $\gamma\delta$  heterodimers did indeed exist and represented a new T cell class. Thus the immunology of  $\gamma\delta$  T cells has been progressing in a “reversed direction,” i.e. from genes to the TCR and from the TCR to a new class of lymphocytes [19]. The four TCR chain gene families ( $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ ) appear strongly conserved across more than 450 million years of evolution of the jawed vertebrates [9, 20]. Since their discovery,  $\gamma\delta$  T cells have been recognized as regular constituents of higher immune systems, with major progress having been made in elucidating both their physiological roles and the nature of their antigenic



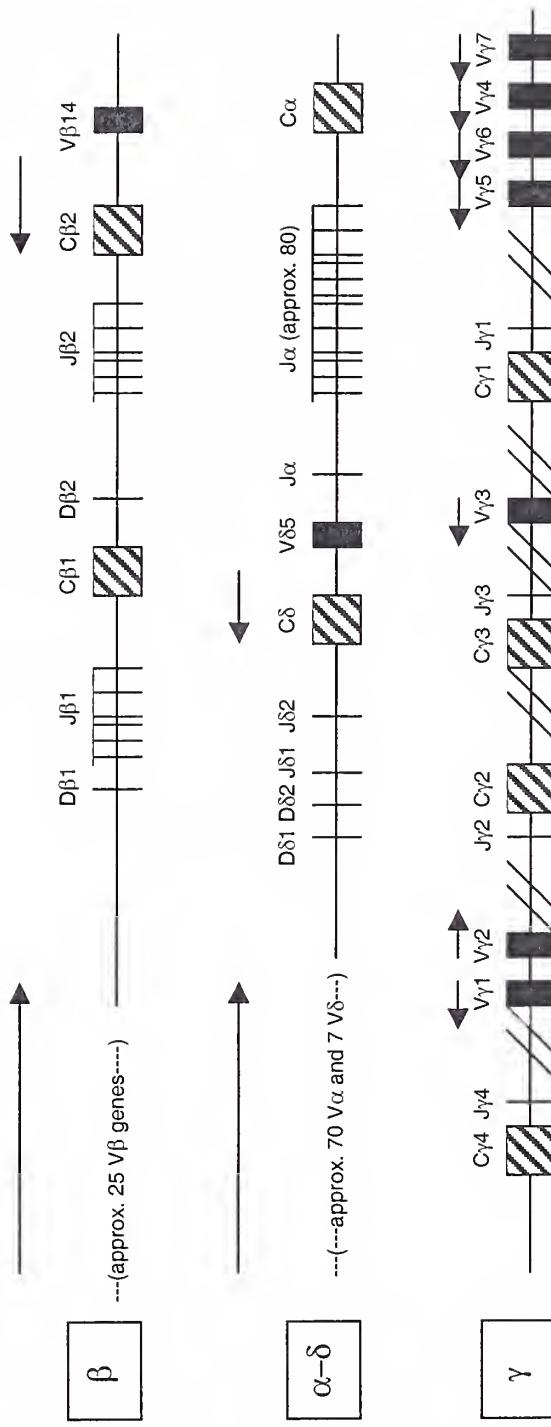
recognition [9]. Yet, a compelling explanation for the evolutionary conservation of  $\gamma\delta$  cells and the strong selective pressure for their retention is still lacking. While much is known about TCR  $\gamma$  and  $\delta$  gene organization, sequence repertoire, and ontogeny, our understanding of the role of these cells in the immune system and the mechanisms by which they function remains at best, only modest [8].

### **$\gamma\delta$ TCR Gene Structure**

Like the  $\alpha\beta$  TCR, the  $\gamma\delta$  TCR is expressed as a heterodimeric cell surface molecule [10, 19]. Cell surface expression of the heterodimer and signaling through the  $\gamma\delta$  TCR also require association with the CD3 complex of transmembrane proteins. Thus, several similarities exist between the overall structure of the  $\gamma\delta$  TCR complex and the  $\alpha\beta$  TCR complex [21]. However, a more detailed analysis of  $\gamma\delta$  TCR structure has revealed substantial differences with  $\alpha\beta$  TCRs as well.

TCR proteins are derived from sets of germline genes containing arrays of coding segments, named variable (V), joining (J), and in the case of  $\beta$  and  $\delta$ , diversity (D) regions [2]. The genomic organization of the TCR genes is shown schematically in Figure 1 [22]. Recirculating  $\alpha\beta$  and  $\gamma\delta$  TCRs exhibit an enormous collective diversity which is generated through a variety of mechanisms during the process of recombination. One mechanism is combinatorial diversity through somatic rearrangement of the genes through the complex of enzymes termed V(D)J recombinase, which brings different coding segments into juxtaposition to produce a functional gene; such gene rearrangement occurs during thymic development and is dependent on Recombinase





**Figure 1. Schematic representation of murine T cell receptor loci.** TCR-V gene segments are shown as solid boxes; -D as bold vertical lines; -J as vertical lines; and -C as hatched boxes. Arrows depict transcriptional orientation. The nomenclature used in this transcript is from Heilig & Tonegawa 1986 [60].



Activating Gene (RAG) products, which are the lymphoid specific components of the V(D)J recombinase [2].

For  $\alpha\beta$  TCRs,  $\sim 100$   $V\alpha$  and  $\sim 50$   $V\beta$  loci allow for  $\sim 5,0000$  different combinations. However, the vast majority of TCR diversity among such recirculating cells is concentrated within the V-J or V-D-J junctional region. The huge diversity potential of the junctional regions of the  $\alpha$  and  $\beta$  genes is calculated to allow  $\sim 10^{15}$  different coding segments [23]. Junctional (CDR3 region) diversity is achieved through the activities of two enzymes: 1) DNA exonuclease, through which the recombining gene segment ends are variably trimmed; and 2) terminal deoxynucleotidyl transferase (TdT), which mediates insertion of variable numbers of non-germline-encoded (N) nucleotides into these junctions. Of note, TdT is known to be expressed particularly abundantly in thymocytes during late fetal thymic ontogeny [24]. This marked TCR heterogeneity allows for the collective capacity of recirculating T cells to recognize a vast array of foreign antigens. The  $\gamma$  chain genes always involve V-J rearrangement, while the  $\delta$  chain genes are assembled via V-D-J rearrangement, frequently involving D-D joining.

Additional, sometimes quite striking diversity is generated by the inclusion of germline-derived nucleotides (P elements), perhaps by transfer of nucleotides to the blunt end of the opposite strand by a novel enzyme prior to trimming, insertion of non-germline encoded nucleotides, and ligation [25]. Collectively, this marked TCR heterogeneity allows for the  $\alpha\beta$  TCR recognition of a vast array of foreign antigens [2].

In marked contrast to the large number of V and J segments in the  $\alpha$  and  $\beta$  loci, the  $\gamma$  locus consists of only four  $J\gamma$  genes, each associated with a  $C\gamma$  gene, and seven  $V\gamma$  genes [22, 26-29]. The  $\delta$  locus, located within the  $\alpha$  locus between the  $V\alpha$  and



$J\alpha$  segments [18], contains approximately 10  $V\delta$  genes, along with two D and two J elements upstream of a single C gene [22]. With such a small number of  $V\gamma$  and  $V\delta$  genes, there are less than 100 different possible combinations of coding segments through somatic rearrangement. However, in contrast to the limited V-gene combinatorial diversity of  $\gamma\delta$  TCR, the diversity potential of the junctional regions of such receptors is actually several orders of magnitude greater than that of  $\alpha\beta$  TCR (estimated to be  $\sim 10^{18}$  different combinations), with almost all the diversity concentrated at the V-J junctions of both  $\gamma$  and  $\delta$  chains [30, 31]. As mentioned previously, the  $\delta$  locus contains two D segments, which can both be used in the same gene. The use of two D segments greatly increases the variability of the  $\delta$  chain, mainly because extra N-region nucleotides can be added at the junction between the two D gene segments as well as at the V-D and D-J junctions [2]. The existence of this extensive junctional diversity in the  $\gamma$  and  $\delta$  chains suggests that the potential repertoire is as large, or perhaps larger, than that of  $\alpha\beta$  cells [23]. However, evidence has shown that  $\gamma$  and  $\delta$  chain diversity may be much more restricted in certain anatomical sites, as will be discussed below.

### **Lineage, Development, and Distribution**

In the fetal mouse thymus, productive rearrangement and surface expression of  $\gamma\delta$  TCR precedes that of  $\alpha\beta$  TCR [31]. CD3+  $\gamma\delta+$  cells appear as early as fetal day 14, and outnumber CD3+  $\alpha\beta+$  thymocytes until about fetal day 17 [10, 32]. Studies have shown that productive rearrangement and expression of  $\gamma$  and  $\delta$  genes during ontogeny is not random but rather is highly ordered, with subsets expressing a strikingly homogeneous TCR arising in “waves” at different times in ontogeny [31, 32]. The first wave expresses



an invariant TCR composed of V $\gamma$ 5 and V $\delta$ 1 [33]. Appearing around fetal day 14, these V $\gamma$ 5/V $\delta$ 1 T cells later become established selectively in the epidermis. After a few days V $\gamma$ 5-bearing cells decline and are replaced by cells expressing the V $\gamma$ 6 gene, also paired with the V $\delta$ 1 chain. In normal healthy mice, these cells colonize the mucosal epithelia of the tongue, vagina, uterus, and lung [21]. In both the V $\gamma$ 5 and V $\gamma$ 6, as well as the V $\delta$ 1, chains of these very early  $\gamma\delta$  thymocytes, there is a virtual complete lack of non-germline encoded (N) nucleotides between the various coding segments, reflecting the absence of the enzyme TdT from these early fetal T cells [24]. After these initial waves, T cells are produced no longer in bursts but in a continuous fashion. By fetal day 17-18, CD3+  $\alpha\beta$  T cells predominate, comprising more than 95% of the thymocyte population [2]. The  $\gamma\delta$  T cells produced at this stage differ in several ways from those of the early waves. They exhibit a more diverse receptor repertoire, for which several different V gene segments have been used, including V $\gamma$ 1, 2, 4, and 7. These particular V $\gamma$  chains are often paired with various V $\delta$  genes, and are later found mainly in peripheral lymphoid tissues rather than in epithelial sites (though they do populate the epithelia of the lung and intestines) [2]. Additionally, in contrast to the early  $\gamma\delta$ + cells, the receptor sequences have abundant N-nucleotide additions, resulting in markedly heterogeneous junctional regions [31].

Despite similarities in the development of peripheral  $\gamma\delta$  T-cell populations, enormous differences exist between species regarding the tissue distributions and overall sizes of  $\gamma\delta$  T-cell subsets [34]. For example, most peripheral  $\gamma\delta$  cells are double negative for CD4 and CD8 [20]. In the periphery, 0.5-10% of peripheral blood T cells in the human and ~3% of T cells in the murine spleen and lymph nodes are CD4-CD8-  $\gamma\delta$  cells [10]. In the chicken, on the other hand, the proportion of  $\gamma\delta$  cells in peripheral blood and



spleen is higher, approaching 30% of CD3+ T cells, with most (~70%) chicken  $\gamma\delta$  cells in the spleen being CD4-CD8+ cells. The remainder are CD4+CD8- cells, as are  $\gamma\delta$  cells in chicken blood [10].  $\gamma\delta$  T cells overall are comparable in numbers to  $\alpha\beta$  T cells in sheep, cattle, pigs, and chickens, while they are rather infrequent in primates and rodents [21]. The biological significance of these differences has yet to be made clear. If  $\gamma\delta$  T-cell subsets are functionally specialized, the differences may reflect varying requirements for these functions among the species. In order to elucidate the physiologic purposes for these cells, it is logical to approach the study of  $\gamma\delta$  T-cell subsets within an organism as separate entities and to attempt to identify and assess their individual functions, as a crucial adjunct to comparing  $\gamma\delta$  and  $\alpha\beta$  T cells in a more global way.

### **Intraepithelial Lymphocytes (IELs)**

By nature, body surfaces have been designed to inherently limit infection. In addition to having rapid-acting innate responses, such as epithelial cells that synthesize and secrete chemokines, cytokines, and anti-microbial peptides (e.g. defensins), epithelia harbor cells of the adaptive immune system that provide local protection [35]. For example, B cells produce secretory immunoglobulin A, which can neutralize pathogens as well as bind endocytosed pathogens during transcytosis across epithelial cells [36]. Additionally, substantial numbers of T cells are constitutively resident within epithelia. Such intraepithelial lymphocytes (IELs) may be among the most abundant T cell subsets given the large surface area of epithelia [35].

IELs are distinct from systemic T cells in several ways. One of the most distinguishing characteristics of IELs is their subset composition. As previously



described, conventional  $\alpha\beta$  T cells are associated with either CD4 or CD8, which consequently guides recognition of antigen in the context of MHC class I or II molecules respectively. IELs, on the other hand, appear to function in the absence of CD4/CD8 $\alpha\beta$  coreceptors [35]. Studies have shown that IELs are usually positive for only CD8 $\alpha$  without CD8 $\beta$  (CD8 $\alpha+\beta-$ ) or negative for both CD4 and CD8 (CD4-CD8 $\alpha\beta-$ ) [33, 37-39]. T cells expressing CD8 $\alpha\alpha$  homodimer are essentially absent from the circulation, whereas they comprise a large fraction of the CD8+ cells which make up >70% of small intestinal IELs [37]. CD4-CD8- “double-negative” (DN) cells are also rare in the systemic circulation, yet they make up >10% of murine small intestinal IELs and the majority of IELs in other compartments [40]. The observation that IELs are not typically associated with CD4 or CD8 coreceptors suggests that IELs may recognize their ligands directly or that they may preferentially recognize nonclassical MHC-like molecules [35]. Additionally, not only are IELs commonly enriched in TCR $\gamma\delta$ + cells, they frequently exhibit markedly limited tissue-associated antigen receptor diversity [20, 21, 41]. Between 35-65% of murine CD8+ small intestinal IELs and virtually all murine epidermal and vaginal IELs express TCR  $\gamma\delta$  repertoires that are specific to their particular site and distinct from those found in the blood [20]. The prevalence in epithelial surfaces of  $\gamma\delta$  cells with extremely limited diversity, together with the relative absence of  $\gamma\delta$  cells from lymph nodes and T cell areas of the spleen, initiated the hypothesis that  $\gamma\delta$  cells do not routinely rely on professional antigen-presenting cells for antigen recognition [20]. Instead, they may perform in a type of “first line of defense” role [42]. This hypothesis proposes that  $\gamma\delta$  cells recognize generic immunological “stress antigens” that are markers of cell infection or transformation rather than unique pathogen-specific epitopes. The



unconventional antigen specificities of IELs are a rapidly-growing area of investigation, which will be discussed later in further detail.

Though CD4+  $\alpha\beta$  T cells are under-represented in many IEL compartments, not all epithelial surfaces demonstrate such distinction [40, 43]. For example, among murine large intestinal IELs, the majority of cells are still CD4 or CD8 $\alpha\beta$ , similar to those found in the systemic circulation [44, 45].

The heterogeneity within IELs and the variability among IEL compartments have led Hayday et al. (2001) to recently propose a simplified categorization of these cells. Type “a” includes the conventional TCR $\alpha\beta$ + cells that are activated within the systemic circulation, recognizing their antigens through MHC restriction. Type “b” cells include TCR $\alpha\beta$ +CD8 $\alpha\alpha$ + and TCR $\gamma\delta$ + IELs that respond to antigens outside of conventional MHC restriction [40]. Several features distinguish type b from type a IELs, including not only their lack of conventional MHC restriction, but also their inability to be reconstituted from peripheral lymph nodes [46-48] and their lack of immunological memory of infection [49-53]. Additionally, serial analysis of gene expression (SAGE) applied directly *ex vivo* to murine TCR $\gamma\delta$ + and TCR $\alpha\beta$ + intestinal IELs has revealed differential gene expression between type a and type b IELs [40, 54]. The similarity of gene expression between TCR $\gamma\delta$ + DN IELs, TCR $\gamma\delta$ +CD8 $\alpha\alpha$ + IELs and TCR $\alpha\beta$ +CD8 $\alpha\alpha$ + IELs indeed forms the basis for their collective type b classification [40]. Of particular note among the genes expressed highly in type a IELs and not in type b is Ly6C, a T cell memory marker. Conversely, only type b IELs distinctively express high levels of Ly49E, a receptor that recognizes MHC class I molecules and inhibits killing by NK cells by overruling the actions of the killer receptors [2, 54]. Such



distinctions provide insight into the different physiological roles played by these IEL subtypes.

### **Dendritic Epidermal T Cells (DETC)**

One of the most intriguing populations of type b IELs are murine epidermis-restricted dendritic T cells. In the early 1980s, cells in the murine epidermis were found to characteristically express the Thy-1 alloantigen, in addition to forming a conspicuous dendritic network among basal layer keratinocytes [55, 56]. Thus, they were initially named Thy-1+ dendritic epidermal cells (DEC). It was soon discovered that the adult epidermis of all normal strains of mice was populated by Ia-, CD4-, CD8- cells that displayed functional properties of T cells. The properties included responsiveness to mitogen and interleukin (IL)-2, secretion of IL-2, and various forms of cytotoxicity [57-59]. The overwhelming majority of Thy-1+ DEC in normal adult epidermis was soon found to be CD3+ in association with a  $\gamma\delta$ -type of TCR; thus they were renamed DETC.

As the DETC antigen repertoire was further explored, it was discovered in 1988 that four independent DETC lines isolated from trunk skin of AKR/J mice expressed TCRs of striking homogeneity [33]. The DETC clones used identical  $\gamma$  coding segments ( $V\gamma 5J\gamma 1C\gamma 1$ )\*, as well as identical  $\delta$  coding segments ( $V\delta 1D\delta 2J\delta 2C\delta$ ). (\*Using the nomenclature for murine  $V\gamma$  gene segments set by Heilig and Tonegawa 1986 [60]). In addition, the junctional regions of both the  $\gamma$  and  $\delta$  chains were remarkably homogeneous, with very little exonucleolytic nibbling or N nucleotide insertions. Quite startling was the realization that these TCRs were identical to those expressed by the first wave of fetal thymocytes. Further studies using both flow cytometric staining and polymerase chain



reaction analysis on freshly isolated DETC from a variety of different mouse strains confirmed this homogeneity of DETC TCRs [32, 61]. Concomitantly, monoclonal antibodies (MoAb) specific for the  $\gamma\delta$  TCR were being used to analyze uncultured epidermal cells from various mouse strains. Stained epidermal cell suspensions using the MoAb by the name of F536, which is specific for  $V\gamma 5+$  TCRs irrespective of the paired  $\delta$  chain, showed that over 90% of the  $CD3+$  cells were  $V\gamma 5+$  [62]. By a different approach, the 17D1 MoAb, which reacts only with cells expressing the  $V\gamma 5$  chain associated with  $V\delta 1/D\delta 2/J\delta 2+$   $\delta$  chains, was used along with anti- $CD3$  to double stain epidermal sheets. These results demonstrated that over 95% of  $CD3+$  cells also stained with 17D1 [63]. Certainly, these results imply that gene expression and somatic diversification in DETC progenitors are strictly regulated, and that DETC recognition of antigen may be extremely limited [33, 64].

Another striking characteristic of DETC is their apparent restriction in tissue distribution, so far having been isolated only in the skin.  $V\gamma 5/V\delta 1+$  cells have not been detected in any peripheral lymphoid tissues, including blood, lymph nodes, or spleen [64]. Additionally, cells with the DETC-type TCR have not been demonstrated in other epithelial sites known to harbor other distinct subsets of  $\gamma\delta$  cells. For example, IELs in gut epithelium have been found to selectively express  $V\gamma 7+$  cells, which, unlike DETC, are not particularly paired with a specific  $\delta$  chain. In addition, both the  $V\gamma 7$  and the paired  $\delta$  chain show considerable diversity in their junctional sequences [65]. The  $\gamma\delta$  T cells of the epithelium of the vagina, uterus, and tongue, on the other hand, are similar to DETC in expressing a monomorphic TCR devoid of junctional diversity, with one of the TCR chains consistently being  $V\delta 1$ . However, they distinctly express the  $V\gamma 6$  instead of



the V $\gamma$ 5 chain [66]. Likewise, the profile of  $\gamma\delta$  T cells in normal mouse lung is distinct from that expressed in the skin, with cells in this location preferentially using V $\gamma$ 2, V $\delta$ 1, and V $\delta$ 6 genes [67].

Although the role of the thymus in the generation of most T cells is well established [2], the involvement of the thymus in the generation of DETC was less clear. Evidence has accumulated over the past ten years that support the derivation of DETC from early fetal thymocytes. First, identical V $\gamma$ 5/V $\delta$ 1 TCRs are seen on the earliest CD3+ fetal thymocytes, which appear around day 12-13 of gestation, and remain the predominant subtype until day 17 [32]. In thymocytes examined after birth, the V $\gamma$ 5 or V $\delta$ 1 coding segments are rarely seen [68]. Second, reconstitution experiments, using either thymus grafts and cell suspensions or purified CD3+ or 17D1+ day 16-17 fetal thymocytes into neonate *nu/nu* (“nude” or athymic) mice (which otherwise lack CD3+ V $\gamma$ 5/V $\delta$ 1+ DETC in the epidermis) showed evidence of gradual accumulation of such cells in the epidermis in a manner consistent with *in situ* proliferation [69]. Interestingly, engraftment with thymic lobes from newborn mice did not result in the appearance of donor-type DETC in the skin of recipients, supporting the fact that V $\gamma$ 5+ cells may not be detectable in the thymus after gestational day 18 [32]. In addition, transfer of CD3- or V $\gamma$ 5/V $\delta$ 1- fetal thymocytes failed to result in epidermal CD3+ V $\gamma$ 5/V $\delta$ 1+ cells. These results indicate that DETC express their V $\gamma$ 5/V $\delta$ 1 genes even before migrating to the skin and are most likely thymic-dependent [64]. Yet these studies have not precluded the possibility that some DETC are also derived independently of the thymus. Epidermis of old nude mice in fact show dendritic CD3+, albeit 17D1-, cells [63]. In addition, grafts of 16-day fetal skin found to initially contain only scattered Thy-1+ CD3- TCR- cells



gradually become populated by skin graft donor-type CD3+ V $\gamma$ 5+ DETC, which suggests an intracutaneous, possibly thymic-independent differentiation [70].

The mechanism by which TCR diversity among DETC remains so limited has yet to be defined. It has been postulated that DETC precursors actually have a limited gene rearrangement potential [71, 72]. However, recent studies have shown that TCR conformation rather than TCR specificity of particular gene segments may in fact be more important for the function or development of lymphocyte repertoires associated with a specific anatomic site, including the murine epidermis [73-75]. Utilizing disruption of the V $\gamma$ 5 gene, Mallick-Wood et al. (1998) analyzed murine epidermal sheets from V $\gamma$ 5-/- and V $\gamma$ 5+/+ littermates with MoAbs to V $\gamma$ 5, TCR $\delta$ , and CD3 $\epsilon$ . As expected, V $\gamma$ 5+ cells were readily detectable in epidermis from V $\gamma$ 5+/+ mice, but not in that from V $\gamma$ 5-/- mice. However, dendritic CD3+ TCR $\delta$ + DETC were present in V $\gamma$ 5-/- mice in numbers indistinguishable from V $\gamma$ 5+/+ controls, implying that other  $\gamma$  $\delta$  TCRs could substitute for V $\gamma$ 5/V $\delta$ 1. Surprisingly, it was discovered that staining with the MoAb 17D1, which ordinarily reacts only with cells expressing both V $\delta$ 1 and V $\gamma$ 5, defining a characteristic DETC TCR conformation [63, 64], demonstrated epidermal sheets from V $\gamma$ 5-/- mice to exhibit cells with the same conformation. This different  $\gamma$  $\delta$  pairing associated with an outwardly normal DETC population was found to be V $\gamma$ 1/V $\delta$ 1. Thus, it appears that, similar to most B cells and  $\alpha$  $\beta$  T cells, epidermal  $\gamma$  $\delta$  cells are associated more with an antigen receptor conformation than with simple linear epitopes specifically encoded by the V $\gamma$  and V $\delta$  gene segments normally used by DETC [73]. In another study [74], V $\delta$ 1-/- mice were shown to have preserved DETC development in relatively normal numbers, with the most frequently used TCR $\delta$  chain being V $\delta$ 6.



A recent study by Ferrero et al. (2001) presents data further supporting this notion that DETC TCR conformation is critical to their development. Through analysis of early fetal thymocytes, the authors found that V $\gamma$ 5 paired with both V $\delta$ 5 and V $\delta$ 6.3 as early as gestational day 16, showing that V $\gamma$ 5 can pair with V $\delta$ s other than V $\delta$ 1. Thus, TCR diversity of intrathymic DETC precursors appears much greater than that observed among DETC in the skin. This observation raises the possibility that only fetal thymic  $\gamma$  $\delta$  T cells expressing an appropriate TCR will localize later in the epidermis [75]. The authors of this study further investigated the importance of  $\gamma$  $\delta$  TCR specificity for DETC migration/localization in the skin by generating a new TCR- $\delta$  transgenic (Tg) mouse, using the V $\delta$ 6.3 segment, which was chosen for its ability to pair with V $\gamma$ 5 in putative DETC precursors of wild-type mice. As expected, fetal thymocytes expressed the V $\delta$ 6.3 transgene in association with V $\gamma$ 5 as early as gestational day 15-16 on both wild-type and TCR- $\delta$ -/- backgrounds, with a normal percentage of DETC being found in the epidermis of wild-type V $\delta$ 6.3Tg mice. Interestingly, virtually all DETC from these wild-type V $\delta$ 6.3Tg mice expressed V $\gamma$ 5 and V $\delta$ 6.3 segments, in contrast to non-Tg littermates. Surprisingly, no CD3+ cells were found in epidermal cell suspensions from TCR- $\delta$ -/- V $\delta$ 6.3Tg mice. With the observation that V $\delta$ 6.3Tg  $\gamma$  $\delta$  T cells indeed reconstitute other  $\gamma$  $\delta$  T cell compartments, including those in the intestine, liver, and spleen, the authors concluded that a second TCR- $\delta$  expressed by wild-type mice, most probably V $\delta$ 1 as shown by their PCR and sequence analysis, is a prerequisite for DETC maturation and/or localization in the skin.

Such evidence for the selective establishment in the skin of  $\gamma$  $\delta$  cells with TCRs of particular conformations raises several possibilities. One hypothesis is that certain DETC



cells mature as a result of selection by specific “DETC selecting ligands” in the fetal thymus and thus that  $\gamma\delta$  T cells may pass through a similar intrathymic selection process as that by  $\alpha\beta$  T cells [75]. Another possibility is that TCR specificity itself directs the migration of  $\gamma\delta$  T cells to the skin. Yet another prospect is that  $\gamma\delta$  T cells express diverse TCR specificities which can all migrate to the skin, but only those having a permissive TCR would be retained and/or locally expand [75]. Such a selection could be attributed to the recognition of cellular antigen(s) within the target tissue, such as any expressed specifically by keratinocytes in the skin in a sort of peripheral positive selection process.

If a particular DETC TCR conformation is critical for DETC development (i.e. migration and/or localization), as suggested by these studies, as opposed to simple linear epitope recognition as encoded by specific TCR gene segments, the question is emphasized as to the specificity of the DETC TCR in terms of ligand recognition and TCR activation. If it is assumed that the different DETC populations found in these experiments serve the same function within the epithelia, it can be concluded that ligand recognition by DETC TCR is not specific to  $\gamma\delta$  pairing. Rather the conformation of the complementarity-determining region (CDR) 3, which, by extrapolation from immunoglobulin (Ig) and TCR $\alpha\beta$ , is likely to be an important contact site for antigen [73], may not be specific to any one epitope. Another possibility is that the DETC ligand(s) may map partly or wholly outside CDR3, still markedly and selectively activating the  $\gamma\delta$  T cells, just as anti-clonotypic antibody and superantigens act on  $\alpha\beta$  T cells *in vivo* [76, 77].



## TCR Specificity/Antigen Recognition by DETC

One of the keys to unfolding the elusive biological functions of DETC lies in discovering the specific ligands of their TCRs and elucidating the manner in which they respond to their ligands. While conventional  $\alpha\beta$  T cells recognize their antigens in the context of highly polymorphic classical MHC molecules guided by their expression of CD4 or CD8 coreceptors [6, 8], the observation that  $\gamma\delta$  T cells, particularly IELs and especially DETC, are usually double-negative lends favor to the idea that IELs may preferentially recognize nonclassical MHC-like molecules. The interactions between  $\gamma\delta$  TCRs and their ligands are believed to be distinct from those directing the interaction between  $\alpha\beta$  TCRs and their ligands [8, 78]. There may be two types of TCR $\gamma\delta$ -mediated T-cell stimulation [6]. The first may be stimulation of  $\gamma\delta$  T cells by antigen-specific ligands that bind to a highly diverse TCR  $\gamma\delta$  site. In this case, ligands would include the antigenic peptide plus a presenting molecule, such as MHC, MHC-like molecule, or CD1, or the protein itself. Primary stimulation would cause monoclonal expansion of  $\gamma\delta$  cells with a unique TCR. The second form of  $\gamma\delta$  T cell stimulation may be through the binding of ligands to an invariant site on distinct TCR  $\gamma\delta$  isotypes [6]. Here, ligands would be more ubiquitous and perhaps highly conserved, such as a heat shock protein (Hsp) peptide [79], that act as generic immunological distress signals rather than unique pathogen-specific epitopes [35, 42].

Of murine and human  $\gamma\delta$  T cells that have been stimulated by peptides in the context of MHC class I or II molecules, some conventional antigen recognition by  $\gamma\delta$  T cells has been shown [80-83]. Yet the majority of  $\gamma\delta$  T cells have been found to be MHC independent. Murine  $\gamma\delta$  T cells have been identified that are under the control of the



MHC class I-like gene Qa-1 within the H-2T locus, with later experiments showing the ability of the Qa-1 molecule to bind Hsp peptides [84-86]. These  $\gamma\delta$  T cells were found to recognize synthetic peptides in a processing-independent fashion [84]. Evidence is indeed increasing that murine  $\gamma\delta$  T cells can recognize surface-expressed proteins directly and independently from antigen-processing and MHC presentation [6]. For example, a herpes virus protein was discovered to stimulate  $\gamma\delta$  T cells directly without need for processing or presentation [87].

Genetic analysis of the  $\gamma\delta$  TCR reveals that the CDR1 and CDR2 regions are highly diverse, unlike that of the  $\alpha\beta$  TCR [8]. With such diversity, it appears that the  $\gamma\delta$  TCR would not accommodate recognition in the context of MHC molecules. Yet, in the past decade, the closely related nonclassical MHC class I molecules T10 and T22 have been identified as ligands for  $\gamma\delta$  T cells, two independently isolated clones called KN6 and G8 [88, 89]. Analysis of the T10/T22 recognition by G8 shows it to be clearly different from MHC class I recognition by  $\alpha\beta$  T cells, with T10/T22 not requiring peptide or any other ligand for cell surface expression or recognition by  $\gamma\delta$  T cells [90, 91]. The T22 or T10 heavy chain need only be associated with  $\beta_2$ -microglobulin ( $\beta_2$ M) in a properly folded and stable heterodimer in order to be recognized by  $\gamma\delta$  T cells and in turn stimulate them [91]. There is no evidence that T10 or T22 present antigen to  $\gamma\delta$  TCR. In fact, the primary sequences of these two nonclassical MHC molecules suggest that the necessary structural features to bind peptide are absent [88, 92]. Structural differences found between T22 and classical MHC class I (class Ia) molecules, with the peptide binding groove being narrower and shorter than that of the conventional MHC molecule, further support the idea that  $\gamma\delta$  and  $\alpha\beta$  TCRs recognize antigens differently.



and that  $\gamma\delta$  TCRs may in fact be more like immunoglobulins in their recognition properties [91]. Interestingly, it has been found that the affinity of the G8 interaction with T10 and T22 is of higher affinity than most  $\alpha\beta$  TCR-ligand interactions, suggesting that these  $\gamma\delta$  T cells may require TCRs with unusually high on-rates in order to antigenically capture these ligands, which are only expressed transiently at the cell surface [90]. Additionally, the dissociation rate of G8 from T10 or T22 has been found to be significantly lower than that for most  $\alpha\beta$  TCR-peptide-MHC interactions [90, 93]. Thus, the threshold at which  $\gamma\delta$  T cells respond to T10 or T22 may be much higher than that required to trigger  $\alpha\beta$  T cells. Such a mechanism may play a key role in preventing the easy activation of  $\gamma\delta$  T cells by self antigens inappropriately.

Additional ligands have recently been identified for human  $\gamma\delta$  T cells: distant MHC class I homologues called MICA and MICB [94, 95]. Unlike MHC class Ia proteins, the expression of MIC proteins in normal tissues is restricted mainly to intestinal epithelium [96]. Functionally distinct from classical MHC class I molecules, MICA and MICB seem to play no role in presenting intracellular antigen [95]. The recently determined crystal structure of MICA supports this notion, as it revealed an inverted peptide binding groove facing the cell essentially incompatible with peptide presentation [97]. Rather than antigen-presenting molecules, they may be functioning as self-antigens that can be stress-induced [95]. MICA and MICB have also been implicated as tumor-associated antigens, with expression being shown in carcinomas of the lung, breast, kidney, ovary, prostate, and colon, with significantly increased frequencies of V $\delta$ 1  $\gamma\delta$  T cells [94]. Both MICA and MICB are recognized by cytotoxic V $\delta$ 1+  $\gamma\delta$  T cells [95]. V $\delta$ 1  $\gamma\delta$  T cell lines and clones derived from the tumors were



shown to recognize these molecules on tumor cell lines and on heterologous and autologous tumor cells. Thus, MICA/B may serve as antigens for V $\delta$ 1  $\gamma\delta$  T cells in a process of tumor immune surveillance [94]. Though it appears that the recognition of these MHC homologues is mediated via TCR reeognition [95], the nature of the interactions is eurious since T cells expressing diverse CDR3 regions are capable of engaging these molecules [95]. It is possible that such a common antigen specificity might enable a subset of V $\delta$ 1  $\gamma\delta$  T cells to respond uniformly to MICA/B, which may act as generic tissue “stress” signals triggered by infection or transformation [94]. No evidence thus far has demonstrated definitive interaetion of MICA/B with  $\gamma\delta$  TCR. More recently, a receptor for MICA has been deteeted on most  $\gamma\delta$  T cells, as well as on CD8+  $\alpha\beta$  T cells and natural killer (NK) cells, identified as NKG2D [98]. It has been shown that engagement of NKG2D can activate eytolytic responses by  $\gamma\delta$  T eells and NK cells against epithelial tumor cells and transfectants expressing MICA. Thus, NKG2D reeognition of MICA and MICB may serve to provide enhancement of innate antitumor activities of NK cells and antigen-specific T-eell responses [98]. Though the MICA/B locus is not conserved in the mouse, and thus no murine MICA/B homologues exist, mice express NKG2D, as well as counterpart NKG2D ligands. These NKG2D ligands have been cloned and found to be MHC class I-like molecules of the Rae-1 family and H60 [99]. Intriguingly, it has been reeently demonstrated that skin cells exposed to carcinogens express Rae-1 and H60 [100]. These molecules are indeed favorable prospects to be DETC-specific ligands.



## Functional Activities of DETC

DETC comprise one of two populations of leukocytes in the epidermis of adult mice, the other being Langerhans cells. As they reside normally in epithelial tissues, DETC live a rather sessile life in close contact with keratinocytes [101]. The biologic roles of  $\gamma\delta$  T cells and DETC in particular, with such curious and marked genetic and anatomic restriction, remain one of the most persistent and obvious questions. Nonetheless, several different functions have so far been described.

### *A. Tissue Maintenance*

Given that DETC exclusively colonize the epidermis, it seems probable that their functions are associated specifically with this tissue. In fact, it was found that epidermal  $\gamma\delta$  T cells do indeed support the growth of keratinocytes through secreting a factor indistinguishable from keratinocyte growth factor (KGF), also referred to as fibroblast growth factor VII (FGFVII) [102, 103]. Interestingly, freshly isolated resting DETC do not produce KGF, while DETC activated with anti-TCR MoAb do express KGF. Other types of epithelial/mucosal  $\gamma\delta$  T cells, such as activated  $\gamma\delta$  IELs from the gastrointestinal tract, but not lymphoid  $\alpha\beta$  or  $\gamma\delta$  T cells, have been found to produce KGF, demonstrating a likely interdependence between  $\gamma\delta$  T cells and their surrounding epithelial cells [102, 104]. Because KGF is also produced by dermal fibroblasts [103], it is possible that KGF production by  $\gamma\delta$  T cells is merely redundant. However, it is more likely that production of this factor by  $\gamma\delta$  T cells becomes important under conditions when fibroblasts are not stimulated, such as in response to signals that are only perceived by  $\gamma\delta$  T cells, and possibly through the  $\gamma\delta$  TCR [21]. Again, the ligand(s) for DETC are not yet known.



Studies have shown that keratinocytes themselves express a ligand for the antigen receptor of DETC. Specifically, Havran et al. (1991) showed that DETC were stimulated to produce lymphokines and to proliferate on contact with freshly isolated skin keratinocytes or a cultured keratinocyte cell line, PAM [105]. Stimulation of DETC could not be detected, on the other hand, after coculture with freshly isolated fibroblasts, splenocytes, or peritoneal exudates cells, showing that DETC can respond to keratinocytes but not to other cells tested. The authors of this study also demonstrated, through antibody inhibition with MoAbs specific for V $\gamma$ 3 TCR and CD3 $\epsilon$  chain, as well as TCR gene transfer experiments, that the responses of DETC to keratinocytes are indeed TCR dependent. With these results showing that DETC TCR can recognize antigens expressed by skin-derived keratinocytes, but with no evidence that DETC are activated *in situ* in normal skin, Havran et al. (1991) proposed that the antigen is induced by “stress” that is associated with dissociation of the tissue or by *in vitro* tissue culture. A potential target may be antigens derived from endogenous Hsps, as most mammalian cells in tissue culture can express increased levels of Hsps [106, 107]. Other studies have consistently provided evidence supporting the ability of injured keratinocytes or transformed keratinocyte cell lines to stimulate DETC. It has been shown that keratinocyte damage *in situ*, caused by contact sensitizing agents, induces localized proliferation of epidermal  $\gamma\delta$  T cells, whereas the same agents do not stimulate the DETC directly [108-110]. These results strongly suggest that DETC recognize keratinocyte damage and function in a form of self-surveillance, allowing such resident invariant  $\gamma\delta$  T cells to respond to a variety of deleterious agents without the need for diverse TCRs that have specificity for foreign antigens [105]. Indeed, this form of immune recognition may



have arisen as one of the earliest forms of protection against damage and disease. Such stimulation of resident DETC by damaged keratinocytes may in turn be capable of promoting keratinocyte growth and wound healing [111]. The interaction between  $\gamma\delta$  IELs and their epidermal neighbors is in fact bidirectional. DETC depend on the presence of IL-7 for their growth and differentiation, which keratinocytes have been shown to produce [35, 112]. Moreover, IL-7 prevents apoptotic cell death that would ordinarily be induced in DETC when exposed to exogenous corticosteroids [112]. Extension studies of different cytokines expressed by keratinocytes at mRNA and/or protein levels have additionally shown epidermal cytokines to regulate the growth of DETC in three different ways: 1) a paracrine mechanism by which keratinocyte-derived cytokines (including IL-7 and tumor necrosis factor [TNF]- $\alpha$ ) promote the growth of DETC; 2) an autocrine mechanism by which DETC-derived cytokines, including IL-2 and IL-4, support their own growth; and 3) a reciprocal pathway in which DETC-derived interferon (IFN)- $\gamma$  modulates the growth of keratinocytes [112]. Thus, the symbiotic relationship between DETC and their neighboring keratinocytes is clearly demonstrated.

Further evidence for the role of  $\gamma\delta$  T cells in epithelial homeostasis comes from studies of intestinal epithelia of mice. IELs are known to regulate enterocyte turnover in monkeys and in guinea pigs [44] and to express enzymes that regulate lipid and cholesterol metabolism [113]. Furthermore, in mice and humans, specific adhesion molecules, namely  $\alpha E\beta 7$  integrin on IELs and E-cadherin on enterocytes promote close contact between IELs and intestinal epithelial cells [114, 115]. This adhesion mechanism may not only promote selective immigration into and retention of intestinal IELs in the gut, but also support functional interactions between both cell types [6]. Mice that are



congenitally deficient in  $\gamma\delta$  T cells have been found to have a reduction in epithelial cell turnover, as well as overall reduced crypt cell numbers and diminished epithelial differentiation [116]. The influence of  $\gamma\delta$  T cells on intestinal epithelial differentiation likely not only affects normal homeostasis but also tissue integrity during infections. For example, TCR $\delta$ -/- mice infected with the parasite *Eimeria vermiformis* (a natural coccidian pathogen of the mouse gut) suffer increased intestinal damage with a pathologically exaggerated pro-inflammatory  $\alpha\beta$  T cell response compared to controls, as well as exacerbated intestinal bleeding, which could be suppressed by adoptive transfer of bulk IELs. This finding indeed supports the notion that the contribution of  $\gamma\delta$  T cells lies in tissue preservation and/or the ability to regulate the consequences of the  $\alpha\beta$  T-cell response [117]. The fact that there is a selective increase in intestinal  $\gamma\delta$  IELs in patients with celiac disease lends further support to the idea that  $\gamma\delta$  T cells maintain epithelial integrity [118]. In this disease, a wheat protein allergy causes CD4+  $\alpha\beta$  T cell-mediated erosion of the small intestinal villi. The dramatic expansion of  $\gamma\delta$  T cells into the intestinal epithelium may reflect the IELs response to a pathognomonic change in the enterocytes, with the purpose to suppress tissue infiltration by systemic T cells. In fact, peak  $\gamma\delta$  IEL representation seems to correlate inversely with disease symptoms [118].

### ***B. Immunoprotection***

Another role that has been implicated for  $\gamma\delta$  T cells is that of recognizing and destroying pathogens directly. Specifically, activated V $\gamma$ 5/V $\delta$ 1+ DETC were found to kill a variety of target cells, including transformed keratinocytes and melanoma tumor



cells [109], as well as to secrete a number of lymphokines [64]. Short-term DETC lines, containing about 95% V $\gamma$ 5+ cells, have been shown to produce small amounts of IL-4 and IFN- $\gamma$  when stimulated with anti-TCR MoAbs, with large amounts of IL-2, while long-term (>1 year) DETC lines produce similar amounts of IL-2 and IFN- $\gamma$ , but no detectable IL-4 [64]. The lymphokine profile of long-term DETC appears to resemble that of CD4+ TCR  $\alpha\beta$ + T<sub>H</sub>1 cells, which typically promote cell-mediated immunity [2]. Studies that have produced profiles of cytokine mRNA of DETC have confirmed these findings, showing that the short-term DETC lines express mRNA for not only IL-2 and IFN- $\gamma$ , but also IL-1 $\alpha$ , IL-3, IL-6, IL-7, TNF- $\alpha$  and  $\beta$ , and granulocyte macrophage-colony stimulating factor (GM-CSF) [119], further supporting their bias away from T<sub>H</sub>2 and toward T<sub>H</sub>1 [20, 120]. On the other hand, other very recent studies (Tigelaar, unpublished observations) of short-term DETC lines have shown that such lines can also express abundant messenger RNA (mRNA) transcripts for the immunoregulatory (down-regulatory) cytokines transforming growth factor (TGF)- $\beta$  and IL-10.

The role of  $\gamma\delta$  T cells in eradicating transformed epithelial cells *in vivo* leads to the hypothesis that  $\gamma\delta$  cells thereby function to not only promote epithelial integrity, but also to prevent dissemination of infected or malignant cells [42]. In fact, the protective role of  $\gamma\delta$  T cells has been implicated in several studies of both infection and malignancy. From early on in the study of  $\gamma\delta$  T cells, it has been noted that  $\gamma\delta$  cells are potently stimulated by mycobacteria, in addition to accumulating at certain sites of microbial replication and selectively expanding in peripheral blood or lymphoid organs in patients suffering from various bacterial or protozoal infections as well as in numerous murine infection models [67, 81, 121-129]. Furthermore, experiments using anti-TCR $\gamma\delta$  MoAbs



have been shown to exacerbate experimental infections of mice with various bacterial and protozoal pathogens [130-133]. The availability of TCR $\beta$  and TCR $\delta$  gene disruption mutants lacking  $\alpha\beta$  (TCR $\beta$ –/–) or  $\gamma\delta$  (TCR $\delta$ –/–) T cells, respectively, allowed for further insights. For example, in a mouse model of experimental listeriosis, both TCR $\delta$ –/– and TCR $\beta$ –/– mice proved rather resistant to infection [134]. However, when the TCR $\beta$ –/– mice were treated with TCR $\gamma\delta$  MoAbs, the mice became susceptible to listeriosis. These findings suggest that  $\gamma\delta$  T cells indeed play a role in antimicrobial immunity; yet, they may be performing primarily compensatory functions that only become salient in the absence of  $\alpha\beta$  T cells [6]. Other studies have suggested an overtly protective function of  $\gamma\delta$  T cells. In a study on infection by *Mycobacterium tuberculosis* (an aerobic, acid-fast bacillus forming exudative and granulomatous lesions), TCR $\delta$ –/– mutants succumbed to the infection while immunocompetent controls did not [135]. Likewise, airway infection of mice by *Nocardia asteroides* (an aerobic filamentous gram-positive bacteria that causes pulmonary infection progressing to abscess and sinus tract formation) leads to 100% fatality in TCR $\delta$ –/– mice, while 100% of wild-type mice clear the infection [136]. As DETC have been found to produce IFN- $\gamma$ , the phenotype exhibited in the TCR $\delta$ –/– mice was likely enhanced by the failed recruitment of neutrophils and macrophages by the  $\gamma\delta$  T cells.  $\gamma\delta$  T cells do appear to play a unique immunoprotective role since the presence of  $\alpha\beta$  T cells in the TCR $\delta$ –/– infected mice was not sufficient to prevent infection and eventual mortality.

Studies on the immunoprotective functions of  $\gamma\delta$  cells using the *Eimeria vermiciformis* infection system on murine intestinal mucosal surfaces have revealed additional interesting observations regarding the biological role of  $\gamma\delta$  cells.  $\alpha\beta$  T cell-



deficient mice revealed a marked susceptibility to primary infection by *Eimeria*, as well as a failure to develop immunity to rechallenge [117]. In contrast,  $\gamma\delta$  T cell-deficient mice did not show any defects in infection or immunity development. Comparison between TCR $\beta$  $^{-/-}$  mice and TCR( $\beta$  X  $\delta$ ) $^{-/-}$  mice which lack all T cells showed the latter to be even more susceptible to infection, implying that though they do not seem to be required by  $\alpha\beta$  T-cell competent mice,  $\gamma\delta$  cells do respond to the infection, as well as play a role in pathogen clearance [117]. A further comparison between IFN- $\gamma$  $^{-/-}$  mice and TCR( $\beta$  X  $\delta$ ) $^{-/-}$  mice showed both models were equally susceptible to infection, lending support to the idea that  $\gamma\delta$  cells may be contributing to infection control through the production of IFN- $\gamma$ . Interestingly, when *Eimeria* infection was studied on mice of different ages, a marked difference in infection susceptibility was found [120]. In young mice, in complete contrast to adult mice, TCR $\beta$  $^{-/-}$  mice show little or no increased susceptibility to primary infection. On the other hand, infection susceptibility is markedly increased in young TCR $\delta$  $^{-/-}$  mice. These results fit together well with the observation that in all vertebrates in which  $\gamma\delta$  cells have been studied,  $\gamma\delta$  cells are disproportionately abundant in young animals, at the body surfaces [120]. Thus, evidence points to an hypothesis of an age-dependent role for  $\gamma\delta$  T cells in which they are more important for controlling primary infections in young animals before  $\alpha\beta$  T cells assume this role [120].

Evidence has shown that  $\gamma\delta$  T cells may also play a role in down-regulating epithelial malignancies. Human intestinal  $\gamma\delta$  IELs have been shown to kill human bowel carcinomas and other intestinal epithelial cell lines [94, 95]. Additionally, TCR $\delta$  $^{-/-}$  mice have been found to be more susceptible to development of colon carcinomas [137]. Most



recently, Girardi et al. (2001) have shown that  $\gamma\delta$  T cell-deficient mice are significantly more susceptible to cutaneous carcinogenesis. Cutaneous malignancy was induced either by inoculation of cell of the squamous cell carcinoma line PDV or by chemical carcinogenesis using intradermal injection of methylcholanthrene (MCA) or applications of dimethylbenz(*a*)anthracene (DMBA) and phorbol ester (12-*O*-tetradecanoylphorbol; TPA). In all three regimens,  $\gamma\delta$  T cell-deficiency promoted cutaneous tumor development. The susceptibility of TCR $\delta$ <sup>-/-</sup> mice indeed indicates that the antitumor effect of  $\gamma\delta$  cells are not simply substituted by that of  $\alpha\beta$  T cells and NK cells [100]. Interestingly, comparison of tumor development via PDV cell injection in wild-type mice, TCR $\delta$ <sup>-/-</sup> mice, TCR $\beta$ <sup>-/-</sup> mice, and TCR $\beta$ <sup>-/-</sup> $\delta$ <sup>-/-</sup> mice showed that 60% of TCR $\delta$ <sup>-/-</sup> mice developed at least one tumor, compared to <20% of control mice. Meanwhile, ~100% of injected sites developed as tumors in TCR $\beta$ <sup>-/-</sup> and TCR $\beta$ <sup>-/-</sup> $\delta$ <sup>-/-</sup> mice, suggesting that  $\alpha\beta$  cells and  $\gamma\delta$  cells each regulate the growth of PDV-caused tumors, but in different manners [100].

In sum,  $\gamma\delta$  T cells appear to exert important effector functions for immunoprotection distinct from that of  $\alpha\beta$  T cells. Possible mechanisms include being directly antipathogenic via cytolytic and/or T<sub>H</sub>1 activities, initiating antipathogenic activities in other cells, such as macrophages or neutrophils, via the action of cytokines, and contributing to immunoprotection through their protection and maintenance of epithelial cells via the production of epithelial growth factors [120].



### ***C. Immunoregulation***

Increasing evidence suggests that another biological role  $\gamma\delta$  T cells may play is in the regulation of other members of the immune system. For example, macrophages are normally activated to produce cytokines, such as TNF- $\alpha$ , which has multiple biological roles including stimulation of dendritic cell migration to lymph nodes and maturation, hepatocyte activation to synthesize acute-phase proteins, and bone marrow endothelium release of neutrophils [2]. Macrophages appear to have markedly suppressed TNF- $\alpha$  production in mice lacking  $\gamma\delta$  T cells, suggesting that  $\gamma\delta$  T cells may play a role in macrophage activation [138]. Additionally, in studies of Gram-negative bacterium-induced septic shock, it has been shown that TCR $\delta$ -/- mice prove more resistant to developing shock [125]. This finding can be explained in light of the fact that Gram-negative bacterial infections provoke abundant TNF- $\alpha$  production, suggesting that the lack of  $\gamma\delta$  T cells may impair TNF- $\alpha$  production and thus allow avoidance of this harmful sequelae [6].

Several studies have specifically demonstrated that  $\gamma\delta$  T cells may play a critical role in the regulation of inflammation, both by controlling the response of activated  $\alpha\beta$  T cells and in the resolution of inflammation. For example, immunocompetent mice treated with anti-TCR $\gamma\delta$  MoAbs show markedly increased activation of  $\alpha\beta$  T cells reflected by IL-2 production and target cell lysis *in vitro* [139]. A murine model of infection by *Listeria monocytogenes* (a facultative intracellular bacterium) shows additional evidence. Here, intravenous or intraperitoneal inoculation of healthy immunocompetent mice resulted in a vigorous inflammatory response with the subsequent development of *Listeria*-specific cytolytic  $\alpha\beta$  T cells, leading to a clearance of infection within 7-10 days



[132, 134]. In mice lacking  $\gamma\delta$  T cells, however, resolution of the inflammatory response was much impaired; instead of small granulomatous lesions around infected hepatocytes which are eventually cleared by *Listeria*-specific  $\alpha\beta$  T cells and activated macrophages, severe liver parenchymal damage ensued, characterized by large necrotic lesions and *Listeria* not confined to individual hepatocytes [132, 134]. In a separate experiment also involving *Listeria* infection, it was further shown that  $\gamma\delta$  T cells tend to increase simultaneously with or after activated  $\alpha\beta$  T-cell responses, in addition to producing IL-10, an inhibitor of activation and growth of  $T_{H}1$  cells [140]. Thus, it was proposed that  $\gamma\delta$  T cells regulate inflammatory responses of  $\alpha\beta$  T cells [141].

Additional studies have implicated that  $\gamma\delta+$  IELs in particular, resident in the skin or the gut, may locally down-regulate  $\alpha\beta$  T cell-initiated inflammatory responses to allergens or pathogens [117, 142-146]. In several studies, the model of contact hypersensitivity to allergens such as 2,4-dinitrofluorobenzene (DNFB) has been used, from which data have been consistent with a down-regulating effect of DETC on conventional T cells (reviewed in [147]). Studies have also reported an ability of antigen-specific  $\gamma\delta$  T cells to down-regulate same allergen-induced contact hypersensitivity, possibly by the killing of allergen-specific  $\alpha\beta$  T cells [148, 149].

A model of graft versus host disease (GVHD) has previously demonstrated the integral role of  $\gamma\delta$  T cells in suppressing autoreactive T cells [145]. In this mouse model, a C57BL/6-derived autoreactive T-cell clone was injected intradermally into the footpad of syngeneic mice. The grafted cells were shown to take residence in the epidermis where they cause histological changes resembling those seen in human cutaneous GVHD [150]. Whereas GVHD in this model is usually self-limiting with spontaneously



recovered epidermis becoming resistant to subsequent induction of GVHD, TCR $\delta$ -/- mice developed cutaneous GVHD that lasted longer than in normal controls [145]. Additionally, these mice did not show any resistance to subsequent cutaneous induction of GVHD, even though the presence of a large population of  $\alpha\beta$  T cells was detected in the place of epidermal  $\gamma\delta$  T cells. When epidermal  $\gamma\delta$  T cells were reconstituted through injections with day 16 fetal thymocytes from normal mice, resistance against GVHD was restored [145]. Such data clearly point to the role of DETC in local epithelial protection and maintenance, such as in the context of autoimmunity.

In these previous studies, none have critically tested the hypothesis that the specific subset of V $\gamma$ 5+ DETC down-regulate cutaneous inflammatory reactions, while other  $\gamma\delta$ + cells do not. Recently, Girardi et al. (2002) examined the physiological consequences of DETC deficiency in several different genetic strains of  $\gamma\delta$  T cell-deficient mice, in addition to conducting adoptive transfer experiments in which FVB. $\delta$ -/- (Taconic) mice received either V $\gamma$ 5+ cells or other subpopulations of  $\gamma\delta$  cells. The study led to several conclusions. The first observation was that a specific phenotype consisting of a spontaneous cutaneous inflammation of the ears in nonobese diabetic (NOD) mice developed by 5-6 weeks of age without any external manipulation. However, only NOD. $\delta$ -/- mice showed this cutaneous phenotype, while NOD wild-type mice did not. Histopathologic examination of NOD. $\delta$ -/- ear skin resembled that of chronic dermatitis: a thickened epidermis, mild intercellular edema, scattered mononuclear inflammatory cells within the epidermis, and a thickened dermis with dilated blood vessels, eosinophils, mononuclear inflammatory cells, and highly increased numbers of mast cells. Baseline ear thickness was shown to be markedly increased.



Intriguingly, this spontaneous dermatitis was strain-dependent. FVB. $\delta$ –/– showed less dramatic but highly significant increases in ear thickness compared to FVB wild-type animals, while C57BL/6. $\delta$ –/– and wild-type mice showed no clinical or histologic evidence of the phenotype [147]. The second conclusion was that the spontaneous dermatitis phenotype appeared to have an inheritance pattern consistent with a single autosomal recessive gene controlling the susceptibility of the NOD. $\delta$ –/– mice. In an analysis of NOD. $\delta$ –/– and C57BL/6. $\delta$ –/– mice, bred to produce F<sub>1</sub>, F<sub>2</sub>, and (F<sub>1</sub> X NOD) backcross (BC) offspring, 24.5% of the F<sub>2</sub> mice and 42% of the BC mice were identified to have the NOD-like phenotype. These incidence rates were consistent with a possible single autosomal recessive gene inheritance, in which case the expected frequency of the phenotype in the F<sub>2</sub> population is 25% and in the BC population 50% [147]. A third conclusion from the study was that allergic and irritant contact dermatitis reactions are enhanced in  $\delta$ –/– mice, but also in a strain-dependent way. FVB. $\delta$ –/– mice showed strikingly augmented ear swelling to both allergic contact dermatitis induced by sensitization and challenge by DNFB and irritant contact dermatitis, induced by application of TPA, while C57BL/6. $\delta$ –/– did not. Finally, the authors found that spontaneous and augmented irritant dermatitis responses in  $\delta$ –/– mice require  $\alpha\beta$  T cells, since  $\beta$ –/– $\delta$ –/– did not show evidence of either, and that V $\gamma$ 5+ DETC are necessary and sufficient for down-regulation of dermatitis. Indeed, FVB. $\delta$ –/– mice reconstituted at 1-3 days old with >98% pure V $\gamma$ 5+ gestational day 17 fetal thymocytes did not show evidence of spontaneous dermatitis, whereas those injected with V $\gamma$ 5– fetal thymocytes had an increased baseline ear thickness comparable to those of FVB. $\delta$ –/– mice [147]. However, because the ears of the V $\gamma$ 5- recipients was found to be only minimally



reconstituted with V $\gamma$ 5-  $\gamma$ 8+ T cells, it could not be concluded that V $\gamma$ 5-  $\gamma$ 8+ cells could in fact play an anti-inflammatory role if they were present in substantial numbers. Yet, in one experiment where it was possible to populate the skin with substantial numbers of  $\gamma$ 8 cells bearing non-DETC TCRs (derived from peripheral lymph nodes of TCR. $\beta$ -/- donors), no down-regulation of  $\alpha$  $\beta$  T cell-induced local inflammation was seen.

The data from this study provide evidence that V $\gamma$ 5+ DETC play a nonredundant, local regulatory role of cutaneous inflammation induced by  $\alpha$  $\beta$  T cells. Yet, there remain many unanswered questions. First, the spontaneous, localized, cutaneous inflammation that develops in particular strains of  $\delta$ -/- mice resembles a chronic dermatitis that may be similar to human atopic dermatitis (AD) [151]. Thus, the phenotype observed in this study may serve as a new animal model for human AD. In fact, a few mouse models have already been proposed for this chronic inflammatory disease, in which many genetic analyses have been undertaken [152-154]. Additional studies evaluating IgE levels and the effects of the environment on development of the dermatitis are needed in order to elucidate the similarities and differences between the spontaneous dermatitis seen in these  $\delta$ -/- mice and human AD. In addition, the need for further detailed genetic analyses is salient in order to clarify any relation between the current model and existing models of chronic cutaneous inflammation mimicking human AD.

### **Relevance of DETC Model to Humans**

The situation in human skin is quite different from that in murine skin. In normal human skin the majority of CD3+ cells (>95%) are localized in the dermis, whereas only 2-5% of CD3+ cells are within the epidermis [155, 156]. Furthermore, in the dermis, up



to 80% of the CD3+ cells possess the  $\alpha\beta$  TCR, whereas only 2-9% bear the  $\gamma\delta$  TCR [155, 157]. Of the 2-5% of CD3+ cells within the epidermis, 60% express the  $\alpha\beta$  TCR and 18-29% the  $\gamma\delta$  TCR [155, 156, 158]. Some even report a proportion less than 0.1% of CD3+ intraepidermal T cells expressing the  $\gamma\delta$  TCR [157, 159]. In addition to the stark difference in prevalence of intraepidermal  $\gamma\delta$ + cells, human skin and murine skin also differ in that the  $\gamma\delta$  T cells in human skin lack the dendritic shape of DETC [160]. The functions of  $\gamma\delta$  T cells in human skin is unknown, and currently, it is generally accepted that a morphologic equivalent of murine DETC does not exist in humans [156, 161]. However, most recently, an analysis of normal human skin has revealed that cutaneous  $\gamma\delta$  T cells express a highly restricted TCR $\delta$  repertoire distinct from that of peripheral blood  $\gamma\delta$  T cells [160]. From separate skin areas, identical dominant TCR $\delta$  rearrangements could be identified. Based on these recent findings, it is indeed conceivable that human cutaneous  $\gamma\delta$  T cells, albeit of a smaller population, represent a functional equivalent of murine DETC.

## B. GENETICS OF ATOPIC DERMATITIS

Allergic diseases, both systemic and site-specific, affect approximately 20-30% of the general population and constitute a major source of suffering, disability and loss of productivity throughout the world. Atopy refers to the predisposition to develop the group of inflammatory diseases which occur following exposure to antigens that are otherwise innocuous, for example, dust mite, pollen, mould and animal dander [162]. Atopic diseases as many other immunological diseases are multifactorial complex traits with a significant impact of both genetic and environmental factors on the outcome of the



disease. The identification of the multiple genetic and environmental factors is confounded by heterogeneity both within a specific atopic disorder, such as asthma, allergic rhinitis, allergic conjunctivitis, and atopic dermatitis, and between the groups of diseases. Thus, symptoms produced may be phenotypically indistinguishable to the clinician even though the genetic predisposition for a specific disorder may in fact result from distinct sets of susceptibility genes in different individuals. Despite these inherent difficulties, there has been remarkable progress towards the identification of the various factors which contribute to the pathophysiology of atopic diseases. During the past 30 years, much of the focus has been on defining the cells and molecules which mediate the allergic reaction [163]. Further elucidation has been achieved through studies of the nature of allergens and from fundamental investigations of the immune response [164]. Several studies involving both extended-family and twin-study approaches have provided clear evidence that both the atopic condition in general and specific allergic disorders have a strong genetic component. Like other complex genetic diseases, however, there is a clear environmental role, making the estimate of the number of genes contributing to the heritability of the condition not at all clear. While there are certainly major genes that control atopy in general, disease-specific genes that determine which disease an atopic individual develops are also likely to exist [165, 166]. Defining such genetic factors will shed light on pathophysiologic mechanisms, which in turn should lead to better treatment strategies. In the analysis of complex traits, however, the genetic analysis is complicated by genetic heterogeneity, phenocopies, gene interactions, and incomplete penetrance.

The marked increase in the prevalence of AD over the past three decades has led investigators to aggressively pursue elucidation of its pathogenesis [167]. AD is



characterized by complex symptoms including a chronically relapsing course, extreme pruritus, and eczematous skin lesions with typical morphology and distribution, which are associated with IgE hyperresponsiveness to environmental allergens [168]. AD is one of the most common skin diseases in children with a family history of atopy, with the prevalence of atopic eczema peaking in the first 5 years of life [169, 170]. Twin studies have helped to elucidate the genetic component to atopic eczema, showing concordance of disease in monozygotic twins (0.72) compared with dizygotic twins (0.21) [171]. Most atopic diseases have a heritability of between 0.60 and 0.72 [172]. In looking for genetic factors that might predispose an individual to atopy, it is logical to examine the pathophysiologic pathways involved in allergic responses to focus the initial search on a few key genes. Very few studies to date have been done on the genetics of atopic dermatitis, with reported findings often being discordant [166]. The genomic regions of interest in the initial studies include chromosome 5q31 (in the region of the genes coding for IL-4, IL-5, IL-9, IL-13, interferon regulatory factor 1, GM-CSF-2, CSF-1 receptor, and platelet-derived growth factor receptor) [173, 174] and chromosome 11q13 (at the locus for the high-affinity Fc $\epsilon$  receptor 1, Fc $\epsilon$ RI) [175-182]. One linkage study using markers on 11q13 flanking the Fc $\epsilon$ RI locus showed negative findings [182], while a later study showed some evidence for support in a subgroup of families with atopic dermatitis [183]. An association between atopic eczema and the presence of a polymorphic restriction site within the mast cell chymase (MCC) gene on chromosome 14q11.2 has been shown in the Japanese population [184], with a proposed second association with a polymorphism in the promoter of IL-4 on chromosome 5q [185]. Most recently, an association between atopic dermatitis and IL-13, which plays an important role in the



induction of IgE synthesis and in the pathogenesis of allergic diseases [186-188] was investigated. A novel IL-13 coding region variant at 4257 bp was found to have a significant association with both high total serum IgE level and AD [189].

An alternative approach involves performing a genome-wide search to identify novel candidate regions [178]. With this approach, linkage analysis studies are performed using microsatellite markers dispersed at roughly equal intervals throughout the genome. LOD scores (log base 10 of the likelihood ratio under the hypotheses of linkage and non-linkage) are determined, which serve as the major criterion of the likelihood that an atopy gene resides at that location in the genome. To date, two genome-wide screens for atopic dermatitis in humans have been reported. The first of these found linkage of AD to chromosome 3q21 [190]. The second screen found linkage of AD or AD and asthma combined to chromosomes 1q21, 17q25, and 20p [191]. All four linkages correspond to known psoriasis susceptibility loci [191], suggesting that the regions contain clusters of genes with general effects on skin immunity and inflammation. It is clear that both approaches, analyzing candidate genes and using genome-wide mapping studies, have yielded potentially important genes which may contribute to genetic predisposition for atopic disease [165].

Inbred mouse strains have proven very valid for the dissection of such complicated phenotypes as atopic dermatitis in the following ways: (1) phenotypic variance between genetically identical individuals has been shown to be caused solely by environmental and stochastic events; (2) segregation of genomic regions or candidate genes can be investigated in large offspring populations; (3) linked chromosomal segments can be further refined with congenic mouse strains, where parts of the candidate



region are introgressed into an identical genetic background; (4) single and combined functions of candidate genes can be investigated by mutation through gene disruption, transgenesis and cross-breeding of such strains; and (5) random mutations within the genome through chemicals can detect novel candidate genes, which are involved in the critical pathways and networks of these complex traits [192]. While several studies have investigated the genetics of atopy-related phenotypes of the respiratory tract in inbred mouse strains [192], mouse models for atopy-related phenotypes of the skin have been rare to date.

Thus far, two genome-wide linkage screens for susceptibility loci for atopic dermatitis have been performed in two different mouse models. The Naruto Research Institute Otsuka Atrichia (NOA) mouse is a mutant mouse strain recently discovered in 1997, having originated from a spontaneously sparsely coated mutant male mouse obtained in 1982 by cross breeding between a female C3H/He mouse and a male ddY (hairy strain) mouse [193]. The NOA mouse is characterized by becoming completely hairless and smooth-skinned in adulthood until the spontaneous development of severe ulcerative skin lesions from the tenth week of age. The animals are housed under barrier-system conditions, periodically subjected to microbiological monitoring, and confirmed to be free of categories A, B, C, and E of microbes and parasites specified for monitoring in mice and rats by the ICLAS Monitoring Center (Asia) [194]. Prevalence rates of these ulcerative skin lesions are 30% by the tenth week of age, increasing to 90% by the 20<sup>th</sup> week of age. In severe cases, the lesions extend to cover almost 20% of the body surface area. Histologically, the ulcerative skin lesions show epidermal desquamation, thickened dermis with a proliferation of inflammatory cells and collagen bundles, and a striking



accumulation of mast cells in and around the lesions [193]. Additionally, NOA mice with ulcerative skin lesions show a significantly increased level of serum IgE compared to NOA mice without ulcerative skin lesions, HRS/J mice (a standard hairless mutant mouse), and two strains of hairy mice, all housed in the same conditions [193]. Analysis of NOA backcross progeny of five different mouse strains revealed a significant association between ulcerative skin lesions and markers in the middle of chromosome 4 [153]. Interestingly, the genetic background of the five parental strains used in the backcross breeding to susceptible NOA mice appeared to have a significant impact on the incidence of skin lesions in the progeny: atopic dermatitis developed three times more often in progeny derived from  $T_{H}2$  responder mice (BALB/cByJ and DBA/2J) than from  $T_{H}1$  responder mice (C3H and C57BL6/J). A search for the modifier genes revealed two chromosomal regions with candidate genes suppressing the development of the NOA atopic dermatitis-like skin disease: in the middle of chromosome 7 and in the telomeric region of chromosome 13 [154]. Both loci correspond to regions of synteny to human chromosomes where linkage to different atopic phenotypes has previously been described (chromosome 5q13 and 11q13). In addition, both regions contain candidate genes for atopic dermatitis. The region on chromosome 7 encodes the gene for IL-16, the mRNA of which has been shown to be upregulated in acute atopic dermatitis and is associated with increased numbers of CD4+ T cells at the site of inflammation [195]. The chromosome 13 region contains the possible candidate gene of phosphoinositide-3-kinase regulatory subunit, polypeptide 1 (Pik3r1), which belongs to a family of enzymes involved in the generation of lipid mediators, which in turn activate a series of intracellular kinases [196].



A second mouse model for human atopic dermatitis is an inbred strain called NC/Nga [152]. NC/Nga mice were originated from Japanese fancy mice (Nishiki-Nezumi) and established as an inbred strain in 1955 [197]. Some Japanese researchers noticed development of spontaneous dermatitis just before or after weaning, but the cause and pathogenesis remained unclear [198]. Upon further investigation, it was observed that these mice spontaneously suffer severe dermatitis in the presence of nonspecified environmental factors. The spontaneous dermatitis developed in NC/Nga mice housed only in air-uncontrolled conventional rooms. In contrast, no skin lesions were detected clinically in mice maintained in specific pathogen-free (SPF) conditions consisting of a laminar filter-air flow enclosure in a bioclean room [198]. Prevalent symptoms of the spontaneous dermatitis included itching, erythema, hemorrhage, edema, crust, drying, and excoriation/erosion hyperplasia of the epidermis region in the face, neck, and/or back, which are all exacerbated by aging. Furthermore, NC mice showed characteristic features of human AD by histopathological examination, such as macrophages and eosinophils invading the dermis, increases and activation of mast cells and lymphocytes, reduction of ceramide, appearance of activated mast cells, and lesional CD4+ T cells [152]. Intriguingly, similarly aged BALB/c mice maintained with conventional NC/Nga mice in the same cage from 7 to 17 weeks of age showed no significant clinical signs or symptoms, suggesting that the dermatitis seen in conventional NC/Nga mice was due not simply to the presence of an environmental factor but also to specific factors intrinsic to the NC/Nga background. Detailed genome-wide mapping studies of NC/Nga backcross mice were subsequently performed in order to identify the genetic loci responsible for human AD using the NC/Nga mouse model. The most significant linkage result was



found on chromosome 9, termed Derm1. Several candidate genes are located near Derm1, including thymus cell antigen 1, theta (Thy1), IL10r, IL18, and cytoplasmic tyrosine kinase (Csk). IL-18 and IL-10 receptor might present the best candidate genes in this region as both have been shown to be important for the propagation of atopic dermatitis, being strongly downregulated in acute lesions of atopic dermatitis in humans [199, 200].

Though the chromosomal regions found in the genome-wide screens for atopic dermatitis in humans do not correspond to the regions identified in the above-mentioned mouse models, further studies may indeed reveal regions corresponding to those found in the human system and will undoubtedly contribute largely to the elucidation of the genes involved in human AD.

### **C. ATOPIC DERMATITIS AND IgE**

As previously mentioned, patients with atopic eczema often have personal or family histories of atopic respiratory disease (asthma and allergic rhinitis) [201-203] and are often characterized by heightened IgE responsiveness [204]. Overwhelming evidence does assert that atopy is indeed a  $T_{H}2$ -mediated disease [165]. The well-known mechanism involves individuals being sensitized to a certain allergen, followed by synthesis of allergen-specific IgE molecules which bind to the high-affinity IgE receptor on mast cells and basophils [2, 37, 165]. Upon re-exposure to allergen, membrane-bound IgE molecules are cross-linked and signals are transduced through antigen-recognition activation motifs, which are found in multiple receptors such as the TCR-CD3 complex, and the Fc receptors [205]. These signals then mediate degranulation of the cells and preformed mediators of allergic inflammation found in the granules are released.



Receptor cross-linking additionally results in the activation of cytokine gene transcription as well as the synthesis of a large panel of newly formed mediators [206]. Atopy could theoretically result from an abnormal regulation of this process at several control points.

The total serum IgE level is highest in patients with atopic eczema compared with patients with asthma, perennial rhinitis, and hay fever [172]. Patients with atopic eczema and atopic respiratory disease often show high serum IgE levels [207], blood eosinophilia [208], increased deposition of eosinophil granule major basic protein in skin lesions [209], and increased number of mast cells in skin lesions [210]. However, patients without personal and family histories of atopic respiratory disease tend to show normal IgE concentration [211-213], normal eosinophil count [208], scant deposition of major basic protein in skin lesions [209], and a normal number of mast cells in skin lesions [210]. Additionally, it has previously been shown that a significant difference exists in the genotype distribution of mast cell chymase, a serine protease secreted by mast cells, between patients with normal IgE concentration and patients with very high IgE concentration [214]. Yet these two groups of patients did not show any difference in the morphological appearance of the skin lesions and the clinical course. Thus, the genetic background of atopic eczema might be heterogeneous and IgE responsiveness may be controlled separately from actual development of the atopic dermatitis phenotype. Further study is indeed needed to clarify the dominant factor determining IgE responsiveness in patients with atopic eczema, as well as the actual role of IgE in the development of atopic dermatitis.

In the proposed mouse models for human AD, the skin lesions that appear in mouse skin are associated with increased IgE levels. Plasma levels of total IgE in



NC/Nga mice are markedly elevated from 8 weeks of age, when mild dermatitis is clinically evident, and increase significantly by 10 weeks, plateauing at 17 weeks, thus correlating with clinical severity of dermatitis [198]. In NOA also, severe ulcerative skin lesions are associated with a significantly increased level of serum IgE [193].

#### **D. GOALS OF PRESENT STUDY**

Although great attention has been focused on the pathogenesis of human AD, the exact etiology remains unclear. Establishment of a suitable animal model is of great importance in elucidating the pathogenesis of this chronic inflammatory skin disease. Girardi et al. (2002) demonstrated a spontaneous, localized, cutaneous inflammation that developed in particular strains of  $\delta$ -/- mice resembled a chronic dermatitis resembling human AD. Thus, the phenotype observed may serve as a new animal model for human AD. As higher IgE levels have been found in human AD patients, the present study sought to determine similarities and differences between the spontaneous dermatitis observed in susceptible NOD. $\delta$ -/- mice and human AD by measuring serum IgE levels in these mice. When compared to IgE levels of mice lacking clinical signs of spontaneous dermatitis, including NOD. $\delta$ -/- housed in SPF conditions, NOD wild-type (w.t.), C57BL/6 (B6) (non-susceptible), and (NOD X B6)F<sub>1</sub> mice, NOD. $\delta$ -/- mice exhibiting spontaneous dermatitis is expected to show significantly higher total serum IgE levels if IgE indeed plays a primary role in the pathogenesis of the spontaneous dermatitis. Likewise, if genotypically identical mice, such as (F<sub>1</sub> X NOD)BC. $\delta$ -/- mice, which show high levels of inflammation are compared to those showing low or absent levels of inflammation, it is expected that higher serum IgE levels will be found among mice with higher levels of inflammation.



As Girardi et al. (2002) showed that the spontaneous dermatitis phenotype is strikingly strain-dependent, existing in NOD but not C57BL/6 mice, a second goal of the present study was to elucidate the genetic role governing the susceptibility to spontaneous dermatitis. Analysis of NOD and B6 simple sequence length polymorphisms at specific loci was performed to clarify the relation between the current model and existing mouse models of human AD, namely NOA and NC/Nga mice. It is hypothesized that the current NOD model will show linkage of spontaneous dermatitis to either of the two major determinant quantitative-trait loci already identified through genome-wide searches to be responsible for atopic dermatitis-like skin lesions in the NOA and NC/Nga mice.

Finally, in the search of ultimately defining the ligand(s) for the DETC-specific V $\gamma$ 5/V $\delta$ 1 TCR, a third goal of the present study was to develop a bioassay in which activation of the V $\gamma$ 5/V $\delta$ 1 TCR could be assessed. A very useful strategy entails isolating the  $\gamma\delta$  TCR in a heterologous cell type. Such  $\gamma\delta$  TCR transfectants have been developed using the rat basophil leukemia (RBL) cell line, using a specific clone called RBL-2H3. This cell line presumably does not express NKG2D, CD8, or other receptors expressed by  $\gamma\delta$  cells, and theoretically avoids the problem of cell activation through an accessory molecule. The background on the development of a RBL bioassay to test TCR activation is briefly discussed below.

## Research Design

### **Development of a bioassay to assess activation through the $\gamma\delta$ TCR**

#### *Fc $\epsilon$ RI vs. TCR $\zeta$ signal transduction pathways*

The RBL-2H3 clone [215] is a subclone from the original Rat Basophil Leukemia cell line [216], and has been widely used to study the signal transduction pathway of the



high affinity receptor it bears for IgE, Fc $\epsilon$ RI. The Fc $\epsilon$ RI structure and signaling cascade resemble those of the  $\alpha\beta$  TCR complex. The Fc $\epsilon$ RI complex is composed of an IgE-binding  $\alpha$  chain, a four-transmembrane  $\beta$  chain, and two disulphide-linked  $\gamma$  chains [217]. Each  $\beta$  and  $\gamma$  subunit contains a single immunoreceptor tyrosine-based activation motif (ITAM), resulting in a total of three ITAMs per Fc $\epsilon$ RI receptor complex. Upon IgE/antigen binding, the  $\beta$  chain-associated Src family tyrosine kinase Lyn phosphorylates the tyrosine residues on the  $\gamma$  chain. Another protein tyrosine kinase called Syk then binds to the phosphorylated ITAMs in Fc $\epsilon$ RI $\gamma$ , resulting in Syk phosphorylation by Lyn. Activated Syk subsequently phosphorylates the membrane-associated cytoplasmic adaptor protein LAT, initiating downstream pathways including phospholipase C- $\gamma$  (PLC- $\gamma$ ) and sphingosine kinase, leading to degranulation.

The signaling cascade utilized by the  $\alpha\beta$  TCR parallels that of the Fc $\epsilon$ RI receptor. As previously described, the  $\alpha\beta$  TCR is a ligand-binding heterodimer closely associated with a CD3 signaling complex [3]. The CD3 signaling complex consists of  $\delta/\epsilon$  and  $\gamma/\epsilon$  heterodimers, in addition to a disulphide-linked TCR $\zeta$  homodimer [4, 218]. TCR $\zeta$  is highly homologous to the Fc $\epsilon$ RI $\gamma$  chain [219], with the significant difference that each TCR $\zeta$  contains three ITAMs compared to a single ITAM on Fc $\epsilon$ RI $\gamma$ . Upon TCR ligation by MHC-presented antigen, the three ITAMs are phosphorylated by the Src family kinases Lck or Fyn [4]. A kinase homologous to Syk, ZAP-70, then binds to the phosphorylated ITAMs and itself is phosphorylated by Lck/Fyn. Activated ZAP-70 subsequently phosphorylates LAT, leading to activation of downstream pathways including PLC- $\gamma$ , ultimately resulting in transcription of specific genes.



The signaling capacity of TCR $\zeta$  and Fc $\epsilon$ RI $\gamma$  has been studied in detail in RBL cells as well as in T cell lines. Early studies showed that  $\zeta$  and  $\gamma$  were each sufficient for full activation of cells [205, 220]. Furthermore, through studies involving the fusion of the transmembrane domain and cytoplasmic tail of  $\zeta$  and  $\gamma$  to the extracellular domains of cell surface antigens such as CD4 and CD8 [205, 220], in addition to studies involving fusion to the extracellular domain of the receptor IL-2R $\alpha$  [221], with expression in T cell lines, the cytoplasmic tail of  $\zeta$  was shown to deliver a more potent signal than  $\gamma$  when expressed in the BW5147 T cell line. When tested in the RBL-2H3 line,  $\zeta$  fusions were found to elicit a stronger response in this system as well. As each  $\zeta$  chain contains three ITAMs compared to one on each  $\gamma$  chain, the stronger response of the  $\zeta$  fusions may be explained by increased strength of signaling due to the greater number of phosphorylated ITAMs in the  $\zeta$  signaling complex. Indeed, it has been shown that the intensity of the response to signaling by recombinant ITAM-containing polypeptide chains is dependent on the number of ITAMs present [222].

### *Serotonin Release Assay*

RBL cells have the ability to release endogenous mediators such as histamine from intracellular granules, in addition to incorporating exogenous molecules and storing them in intracellular granules. Upon binding of antigen/IgE complexes to Fc $\epsilon$ RI, they are activated to release the content of their secretory granules. The original RBL lines secreted very low levels of histamine upon antigen/IgE complex binding to Fc $\epsilon$ RI [216]. While some subclones, including RBL-2H3, did release histamine upon stimulation as detected by ELISA, the histamine content was significantly lower than that of normal



mast cells. A more sensitive degranulation assay was subsequently developed [223]. In this assay system, RBL-2H3 cells incorporate  $^3\text{H}$  5-hydroxytryptamine (serotonin) into their secretory granules via incubation with the radioactive substance overnight. The activated cells release the content of their secretory granules, including their endogenous histamine and the exogenously loaded  $^3\text{H}$  serotonin. The percentage of released radioactivity is easily measured by scintillation counting.

### ***TCR Expression in the RBL System***

The RBL-2H3 line was used in early attempts to produce a soluble  $\alpha\beta$  TCR for crystallographic analysis and measurements of the affinity between TCR  $\alpha\beta$ , MHC, and antigen [224]. The extracellular domains of the  $\alpha$  and  $\beta$  chains of the T cell hybridoma 2B4 TCR were fused with the transmembrane and cytoplasmic tail of the TCR  $\zeta$  chain, and were stably expressed as  $\alpha\text{-}\zeta$  and  $\beta\text{-}\zeta$  chains on the cell surface. The cytoplasmic domain of  $\zeta$  induces cell activation when attached to heterologous proteins that are cross-linked by antibody [205, 220, 221]. Thus, the ability of an  $\alpha\text{-}\zeta/\beta\text{-}\zeta$  heterodimer to bind antigen-MHC could be tested by exposure of the cells that expressed this heterodimer to antigen-pulsed presenting cells. In addition, the  $\zeta$  transmembrane domain induces disulfide dimerization when attached to heterologous proteins [205, 220]. The  $\zeta$  constructs, even in the absence of the CD3 complex, demonstrated the ability to signal upon exposure of the transfectants to cells that presented the appropriate antigen-MHC complex, with activation measured by release of  $^3\text{H}$  serotonin [224]. Engel et al. (1992) also demonstrated efficient transport to the cell surface of  $\alpha$  and  $\beta$  chains that was dependent on disulfide-linked heterodimer formation induced by the  $\zeta$  chain [224].



With the evidence of strong signaling capacity through the cytoplasmic tail of  $\zeta$ , along with previous studies demonstrating the ability to stably and efficiently express functional  $\zeta$  fusion constructs on the RBL cell surface, the RBL system provides an attractive method for studying  $\gamma\delta$  TCR specificity. In this system, the TCR can presumably be isolated in order to search for its ligand(s) since rat basophil cells should not express molecules that could bind ligands on mouse target cells. Additionally, the serotonin release assay is a simple yet highly sensitive method in which to measure activation through the  $\gamma\delta$  TCR. While traditional T cell activation assays measure proliferation or IL-2 release after 24 hours of activation or more [205, 220], serotonin release has the additional advantage of allowing measurement after only 45 minutes of activation. Once stably expressed on the RBL cell surface,  $V\gamma5$  TCR $\zeta$  and  $V\delta1$  TCR $\zeta$  chimeric constructs co-transfected into RBL-2H3 cells should have the ability to stimulate signal transduction upon TCR engagement with ligand, leading to degranulation and serotonin release, thus providing a system through which the DETC TCR ligand(s) can be identified.



## II. MATERIALS AND METHODS

### A. TOTAL SERUM IgE MEASUREMENTS

#### 1. Animals

C57BL/6 (B6) wild-type ( $\delta$ +/+, containing normal  $\gamma\delta$  T cells) and non-obese diabetic (NOD/LtJ) wild-type mice purchased from The Jackson Laboratory (Bar Harbor, Maine, USA) have been maintained at the animal facilities of Yale University. B6 mice deficient in  $\gamma\delta$  T cells (TCR. $\delta$ −/−) (The Jackson Laboratory; Bar Harbor) were backcrossed to NOD mice 14 generations to produce NOD. $\delta$ −/− mice used in this study. For genetic analyses, (NOD X B6)F<sub>1</sub>. $\delta$ −/− and (B6 X NOD)F<sub>1</sub>. $\delta$ −/− offspring were used, produced by mating NOD. $\delta$ −/− mice with B6. $\delta$ −/− mice using both sexes in each strain. In addition, F<sub>2</sub>. $\delta$ −/− offspring generated by intercrossing F<sub>1</sub>. $\delta$ −/− mice, as well as (F<sub>1</sub> X NOD)BC. $\delta$ −/− and (NOD X F<sub>1</sub>)BC. $\delta$ −/− mice produced by backcrossing F<sub>1</sub>. $\delta$ −/− mice to NOD. $\delta$ −/− mice (again using both sexes), were used. Mice were kept in two different housing conditions from birth. Conventional, non-ventilated cages (CNVC) were filter-topped cages with sterilized food and water, along with autoclaved corncob bedding, changed at least once a week. Individually-ventilated cages (IVC) were also filter-topped cages with sterilized food, water, and corncob bedding, but in such cages room air is changed ~17 times per hour. In such a setting, bedding remains substantially drier and requires changing only every 10-14 days. Repeat testing of mice in both housing conditions failed to identify known bacterial pathogens, mites, or pinworms on the skin.

Serum IgE samples were collected from the following (number of) mice in the specified housing environments: (10) B6 wild-type housed in CNVC, (31) B6. $\delta$ −/− housed in CNVC, (6) NOD wild-type housed in CNVC, (7) NOD wild-type housed in



IVC, (40) NOD. $\delta$ –/– housed in CNVC, (26) NOD. $\delta$ –/– housed in IVC, and (24) (B6 X NOD)F<sub>1</sub>. $\delta$ –/– and (NOD X B6)F<sub>1</sub>. $\delta$ –/– housed in CNVC. The number of samples for each type of mouse and housing environment was determined by the number of mice available within the same age range at the time of testing and from which at least 50  $\mu$ l of serum was able to obtained. Additionally, serum was collected from (F<sub>1</sub> X NOD)BC. $\delta$ –/– and (NOD X F<sub>1</sub>)BC. $\delta$ –/– mice housed in CNVC, in order to compare total IgE levels in (32) mice with a high level of inflammation with (33) mice showing a low level of inflammation. High inflammation mice were defined by selecting the first 15 males and first 17 females from a list in which all mice (121 males and 114 females) were ordered from highest to lowest according to their mean baseline ear thicknesses (inches  $\times 10^{-3}$ ) measured by an engineer's micrometer. Low inflammation mice were defined by selecting the last 16 males and last 17 females from the same list.

## **2. Serum collection**

Sera were collected from mice at age 11-16 weeks. Mice were anesthetized by placement in an anesthetic jar containing Metofane on gauze squares under screening. Blood was collected from the retro-orbital sinus by gently inserting and twisting the tip of a glass capillary Pasteur pipette below the eye between the globe and lower eyelid. Blood was transferred into a labeled 1.5 ml plastic microcentrifuge Eppendorf tube using a pipette bulb to carefully blow out the sample. A volume of approximately 0.25 ml was obtained from each animal. Gentle manual pressure with a gauze pad was applied to the site of collection on each mouse for a few seconds until bleeding was completely stopped. For some mice, blood was collected via cardiac puncture as a terminal collection method using a 1 ml syringe and 22 gauge, 1-inch needle, inserted through the



left thoracic wall at the point where heartbeat was felt. Blood collection was followed by immediate euthanization of the animal by cervical dislocation while under anesthesia as outlined by the Yale University Animal Resources Center [225]. Blood samples were allowed to clot for 4-6 hours at 4°C, were then centrifuged for 5 minutes at 1200 rotations per minute (rpm), and sera were collected into separate clean 1.5 ml plastic Eppendorf tubes using a 1000  $\mu$ l pipetman (Gilson), transferring minimal amounts of cellular product. Samples were centrifuged for another 5 minutes at 1200 rpm. Sera were then transferred into clean 1.5 ml plastic Eppendorf tubes and stored at -4°C until use, at which time samples were thawed at room temperature and kept on ice.

Ear phenotypes and baseline thicknesses of the specific animals used for the measurement of serum IgE levels were assessed on the day of serum collection by Robert Tigelaar, M.D., in which presence/absence of dermatitis was recorded, as well as baseline ear thicknesses using an engineer's micrometer.

### **3. Antibody measurement by Enzyme-Linked Immunosorbent Assay (ELISA)**

For determination of total serum IgE levels, 96-well microtiter plates (Nalge Nunc International, Rochester, New York, USA) were coated with sheep anti-mouse IgE (15.9 mg/ml, 1:000 dilution; The Binding Site, Birmingham, United Kingdom) in borate-buffered saline (pH=8) at 4°C overnight. The following steps were performed, with plates being washed 2 times with phosphate buffered saline (PBS)/0.05% Tween-20 (20mM Tris, 150 mM NaCl, 0.1% tween) and 1 time with PBS between each step. Blocking was performed with 1% bovine serum albumin (BSA) in borate saline (BSA-BS) for 1 hour at 37°C, after which sera were diluted serially (starting at a 1:10 dilution) in 1% BSA-BS in replicate (two for each dilution) wells and incubated for 2 hours at



37°C. This was followed by incubation for 1 hour at 37°C with an optimal dilution of biotin-labeled secondary rat anti-mouse IgE antibody (1 mg/ml, 1:2000 dilution; Clone LO-ME-3, BioSource International). Streptavidin-conjugated horseradish peroxidase (1.25 mg/ml, 1:5000 dilution; Zymed Laboratories, San Francisco, California, USA) was added for a 30-minute incubation at 37°C, followed by addition of 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution (DAKO Corp., Carpinteria, California, USA) and termination of the reaction with 6N HCl. Color change was read at 450 nm on a microplate ELISA reader (Bio-Tek Instruments Inc., Winooski, Vermont, USA). Antibody concentrations were calculated using DeltaSoft™ software by comparison to a mouse IgE standard (starting concentration 100 ng/ml; Monoclonal Anti-dinitrophenyl (DNP) Clone SPE-7 Purified Mouse Antibody, Sigma, St. Louis, Missouri, USA).

#### **4. Statistical analysis**

Statistical significance was determined using a standard Student's unpaired two-tailed *t*-test [226]. A *p*-value of < 0.05 was considered significant.

### **B. GENETIC ANALYSIS**

#### **1. Animals**

To understand better the genetics governing the susceptibility to spontaneous dermatitis (SpD), the (F<sub>1</sub> X NOD)BC.δ<sup>-/-</sup> and (NOD X F<sub>1</sub>)BC.δ<sup>-/-</sup> mice produced by backcrossing F<sub>1</sub>.δ<sup>-/-</sup> mice to NOD.δ<sup>-/-</sup> mice (using both sexes as described above) were used in the analysis of NOD and B6 simple sequence length polymorphisms (SSLPs) at certain loci. As described in the study by Girardi *et al.* (2002), susceptible ("NOD-like") mice were identified by an increased baseline ear thickness of  $\geq$  5 SDs above the average



means for “resistant” B6.δ<sup>-/-</sup> and F<sub>1</sub>.δ<sup>-/-</sup> mice; i.e., BC females with an ear thickness of > 14.0 (in. X 10<sup>3</sup>), and BC males with an ear thickness > 15.5 were classified as susceptible. A total of 99 of 235 (42%) BC mice had the NOD-like phenotype using these criteria.

## 2. DNA Extraction

Livers were removed from euthanized donors (using either cervical dislocation or Metofane anesthesia overdose) from each of the 99 (F<sub>1</sub> X NOD)BC.δ<sup>-/-</sup> and (NOD X F<sub>1</sub>)BC.δ<sup>-/-</sup> mice identified to have obvious SpD by the above criteria. Using the DNeasy protocol for animal tissues (DNeasy® Tissue Kit, QIAGEN Inc.; Valencia, California, USA), approximately 25 mg of tissue was cut up into small pieces, placed into a 1.5 ml microcentrifuge tube, and incubated with proteinase K and tissue lysis buffer at 55°C until tissue was completely lysed, with occasional vortexing to disperse the sample. Complete digestion was achieved with overnight incubation, i.e. typically 15-18 hours. Samples were subsequently treated with 1 µl of RNase A (100 mg/ml) and incubated at 37°C for 1 hour. Direct cell lysis was performed using the optimized buffer system as outlined by the QIAGEN protocol, and lysates were loaded onto the DNeasy mini column. During a brief centrifugation, DNA was selectively bound to the DNeasy silica-gel membrane as contaminants passed through. Remaining contaminants and enzyme inhibitors were removed in two efficient wash steps and DNA was then eluted in Buffer AE, provided in the QIAGEN DNeasy® Tissue Kit, and was ready for use.

DNA yield was determined for each sample by measuring the concentration of DNA in the eluate by its absorbance at 260 nm using a spectrophotometer. An A<sub>260</sub> of 1 (with a 1 cm detection path) corresponds to 50 µg DNA per milliliter water. Water was



used as a diluent when measuring DNA concentration since the relationship between absorbance and concentration is based on extinction coefficients calculated for nucleic acids in water [227]. The spectrophotometer was always calibrated with distilled water. An example of the calculations involved in DNA quantification is shown below:

Volume of DNA sample	= 100 $\mu$ l
Dilution	= 20 $\mu$ l of DNA sample + 180 $\mu$ l distilled water (1:10 dilution)
Measure absorbance of diluted sample in a 0.2 ml cuvette:	
$A_{260}$	= 0.2
Concentration of DNA sample	= 50 $\mu$ g/ml $\times A_{260} \times$ dilution factor = 50 $\mu$ g/ml $\times 0.2 \times 10$ = 100 $\mu$ g/ml
Total amount	= concentration $\times$ volume of sample in ml = 100 $\mu$ g/ml $\times 0.1$ ml = 10 $\mu$ g of DNA

The ratio of the readings at 260 nm and 280 nm ( $A_{260}/A_{280}$ ) was calculated for 46 random samples to provide an estimate of the purity of DNA with respect to contaminants that absorb UV, such as protein. When absorbance is measured in 10mM Tris·Cl, pH 8.5, pure DNA has an  $A_{260}/A_{280}$  ratio of 1.8-2.0. The  $A_{260}/A_{280}$  ratio is influenced considerably by pH, and since water is not buffered, the pH and resulting ratio can vary greatly (DNeasy® Tissue Kit Handbook, QIAGEN Inc.). Of the samples whose absorbances were all measured in water, the  $A_{260}/A_{280}$  ratios ranged from 1.35 to 3.41. With the exception of 4 samples, all samples had ratios within the 1.5-2.0 range.



### 3. Primers

As previously discussed, genome-wide microsatellite mapping studies in two other distinct mouse models of “atopic dermatitis-like” disease have recently mapped major susceptibility intervals controlling dermatitis to a region on chromosome 9 in NC/Nga mice [152] and on chromosome 14 in NOA mice [153]. In the NC/Nga (NC) model, linkage disequilibrium between dermatitis and various chromosome-specific microsatellite markers was examined in backcross progeny between (NC X MSM/MS)F<sub>1</sub> and NC, MSM/MS being an inbred strain from Japanese wild mice, *Mus musculus molossinus*. To map major quantitative trait loci (QTLs) for dermatitis, genotyping data on each marker were analyzed by the  $\chi^2$  test.  $\chi^2$  results were evaluated by the values for suggestive linkage ( $\chi^2 \geq 8.7$ ) and for significant linkage ( $\chi^2 \geq 15.2$ ) [228, 229]. The analysis revealed that the locus of the major determinant was tightly linked to *D9Mit163*, *D9Mit72*, *D9Mit143*, *D9Mit103*, *D9Mit207*, and *D9Mit209*, as indicated by these markers showing the highest and most significant  $\chi^2$  values ( $\chi^2 = 16.4$ ). The position of the six reported loci was at 33.0 centiMorgans (cM) and 34.0 cM. Therefore, in order to determine if susceptibility to SpD in NOD. $\delta$ –/– mice is controlled by a locus in the same interval, the *D9Mit105* locus at position 33.9 cM was chosen to be analyzed in the (F<sub>1</sub> X NOD)BC. $\delta$ –/– and (NOD X F<sub>1</sub>)BC. $\delta$ –/– mice. A PCR primer set which distinguishes NOD from B6 SSLPs specific for this locus (Map Pairs) was synthesized at Yale University Howard Hughes Medical Institute/Keck Oligonucleotide Synthesis Facility (Oligoz-R-Us). The primer sequences were as follows: 5'-ACA GAG AAA GGA CAG AGA TCC TG-3' and 5'-TAT CAA ATT GGG AGT CAT TTA TGG-3'. The expected size of the PCR product was 145 base pairs (bp) in the B6 genome and 116 bp in the



NOD genome, i.e. a size difference of 29 bp between the two PCR products (see Table 1) capable of readily being visualized after electrophoresis through a 3% agarose gel.

Locus	Genetic Position (cM)	C57BL/6	NOD	Difference (bp)
<i>D9Mit105</i>	33.9	145	116	29
<i>D14Mit262</i>	37.2	124	154	30

**Table 1. Expected size (in bp) of PCR product using primer sets (Map Pairs) distinguishing B6 from NOD SSLPs.** *D9Mit105* was selected based on the results by Kohara *et al.* (2001) demonstrating significant linkage between dermatitis in NC/Nga mice and markers on chromosome 9 at loci in positions 33.0-34.0 cM. *D14Mit262* was selected based on the results by Natori *et al.* (1999) showing linkage between ulcerative skin lesions in NOA mice and markers on chromosome 14 in the vicinity of 32.5-40.0 cM.

In the NOA model, linkage analysis was performed in NOA backcross progeny, generated by first crossing NOA mice with five other murine strains (B6, IQI, C3H/HeJ, DBA/2J, and BALB/cByJ) and then breeding backcross animals. Using microsatellite markers selected at 10- to 20-cM intervals, Natori *et al.* (1999) scanned the entire genomes of 559 backcross offspring from the five parental strains. Linkage analysis revealed a significant association between ulcerative skin lesions and markers on murine chromosome 14. Statistical analysis revealed that the critical region was assigned to the vicinity of *D14Mit236*, at position 32.5 cM ( $\chi^2 = 86.89$ ;  $P < 0.001$ ), and *D14Mit160*, at position 40.0 cM ( $\chi^2 = 86.62$ ;  $P < 0.001$ ). To determine if susceptibility to SpD in NOD.δ-/- mice is controlled by a locus in the vicinity of these identified loci, the *D14Mit262* locus at position 37.2 cM was chosen to be analyzed as this locus is approximately in the center of the interval between *D14Mit236* and *D14Mit160*. A PCR primer set distinguishing NOD from B6 simple sequence length polymorphisms (SSLPs) at *D14Mit262* was identified (Map Pairs) and synthesized as follows: 5'-CAG AAG



GGA AAT CTT AAA ATG AGG-3' and 5'-CAA CTT AGA TGC ATA CAC AGT TTG C-3'. The expected size of the PCR product was 124 bp in the B6 genome and 154 bp in the NOD genome, i.e. a size difference of 30 bp between the two PCR products (see Table 1).

#### **4. Polymerase Chain Reaction (PCR) Amplifications**

Genomic PCR amplifications were performed on all 99 (F<sub>1</sub> X NOD)BC.δ-/- and (NOD X F<sub>1</sub>)BC.δ-/- samples, each sample in two separate reactions, one with primers for *D9Mit105* and one with primers for *D14Mit262*. PCR amplifications were performed in 50-μl reaction mixtures, consisting of 80 ng of genomic DNA, 25 pmol of each primer, 5 μl of 10X PCR Reaction Buffer (Roche Diagnostics Corporation, Indianapolis, Indiana, USA), 5 μl of 1mM dNTPs, 0.5μl of *Taq* Polymerase (5 units/μl, Roche), and 31.5 μl of double-distilled water (ddH<sub>2</sub>O). PCR conditions included amplification at 94°C for 5 minutes, followed by 30 cycles of melting at 94°C for 60 seconds, annealing at 54°C for 60 seconds, and an extension of 72°C for 60 seconds. A final extension for 7 minutes at 72°C ended the process. All PCR reactions were performed in a DNA Thermal Cycler 480 (Perkin Elmer Cetus, Norwalk, Connecticut, USA). PCR products were electrophoresed on 3% agarose gels in 1X TAE (0.04 M Tris-acetate, 0.001 M ethylenediamine tetraacetic acid {EDTA}) buffer run at 60-80 volts, stained with ethidium bromide, and visualized under ultraviolet irradiation.

#### **5. Statistical analysis**

The number of recombinant genotypes (heterozygous, NOD/B6, versus homozygous, NOD/NOD) for each marker locus was calculated. Genotyping data on



each marker were analyzed by the  $\chi^2$  test. Suggestive linkage was defined by a  $\chi^2$  value of  $\geq 8.7$  and significant linkage by a  $\chi^2$  value of  $\geq 15.2$  [228, 229].

## **C. CONSTRUCTION OF V5-J1-C $\gamma$ 1/V1-D2-J2-C $\delta$ T-CELL RECEPTOR**

### **1. Generation of Expression Construct**

#### **a. DETC cDNA production from mRNA**

Messenger RNA (mRNA) was extracted from dendritic epidermal T cell (DETC) clones previously established by Julie Lewis, Ph.D. and Robert Tigelaar, M.D. (Departments of Dermatology and Immunobiology at Yale University). Clones #7 (named 30E12), #9 (30B9), #12 (3G8), #15 (30F4), #16 (10F1), and #18 (210C11) were used, all of which were established by limiting dilution of interface epidermal cells from C57BL/6-TdT transgenic mice, stimulated initially with 10 U/ml IL-2 + 2  $\mu$ g/ml concanavalin A (ConA) + 1  $\mu$ g/ml indomethacin, followed by expansion and twice-weekly feeding with 5 U/ml IL-2 (Lewis, personal communication). All DETC clones had previously been sequenced by Tigelaar, with clones #7, #9, and #12 shown to have the canonical V $\gamma$ 5 chain, and clones #15, #16, and #18 shown to have the canonical V $\delta$ 1 chain (Tigelaar, personal communication). Using reverse-transcriptase PCR (RT-PCR), DETC copy DNA (cDNA) was generated from the mRNA by Tigelaar, as outlined by the QIAGEN OmniScript RT kit protocol (QIAGEN Inc.; Valencia, California, USA). Briefly, 2  $\mu$ l of Oligo-dT primer (10 mM) was added to 3  $\mu$ l of each RNA sample and incubated for 7 minutes at 68°C in the DNA Thermal Cycler 480. To each sample, 15  $\mu$ l of the following mix was added: 2  $\mu$ l of 10X Buffer RT, 2  $\mu$ l of 5mM dNTPs, 1  $\mu$ l of RNase inhibitor (10 units/ $\mu$ l), 1  $\mu$ l of OmniScript RT, and 9  $\mu$ l of RNase-free water.



Each 20  $\mu$ l reaction was carried out at 37°C for 60 minutes, followed by placement on ice when reactions were done.

### **b. Amplifying V $\gamma$ 5 and V $\delta$ 1 chains from DETC cDNA**

Three  $\mu$ l of cDNA from clone #7, #9, and #12 were pooled together. Similarly, 3  $\mu$ l of cDNA from clone #15, #16, and #18 were pooled together. Using 2  $\mu$ l of pooled cDNA for each reaction, the extracellular domains of V $\gamma$ 5 and V $\delta$ 1 from the DETC TCR were amplified using the primers below (see Figure 2A). The 5' primers added an Xho I restriction site immediately before the leader sequence of V $\gamma$ 5 and V $\delta$ 1. The 3' primers added a Bgl II restriction site directly after the cysteines involved in the interchain disulphide bond, allowing for fusion to the thrombin linker region and CD3 $\zeta$  (see Figure 2B). Briefly, the PCR amplifications were performed in 50- $\mu$ l reaction mixtures, consisting of the following: 2  $\mu$ l of pooled DETC cDNA (5 ng/ $\mu$ l), 25 pmol of each primer, 5  $\mu$ l of 10X *Pfu* Reaction Buffer (Roche Diagnostics Corporation, Indianapolis, Indiana, USA), 5  $\mu$ l of 1mM dNTPs, 0.5 $\mu$ l of *Pfu* Polymerase (2.5 units/ $\mu$ l, Roche), and 33.5  $\mu$ l of ddH<sub>2</sub>O. PCR conditions included amplification at 94°C for 3 minutes, followed by 34 cycles of melting at 94°C for 45 seconds, annealing at 55°C for 60 seconds, and an extension of 72°C for 5 minutes. A final extension for 5 minutes at 72°C ended the process. All PCR reactions were performed in a DNA Thermal Cycler 480. V $\gamma$ 5 and V $\delta$ 1 cDNAs were purified from 1% agarose 1X TAE gels using the protocol from the QIAEx®II Gel Extraction Kit (QIAGEN Inc.; Valencia, California, USA). V $\gamma$ 5 and V $\delta$ 1 cDNAs (80 ng in a 24  $\mu$ l volume reaction) were then sequenced to confirm the correct sequence for each chain.



**Figure 2A. Primers used to generate TCR- $\zeta$  fusion constructs.** Restriction sites added and start codons are shown as labeled. The consensus Kozak sequence CCACC was added before the ATG.

5' V $\gamma$ 5 primer

Xho I site      Start Codon  
 5'- CCG **CTC GAG** CCA CCA **TGT** CAA CCT CTT GGC TTT TTC -3'

3' V $\gamma$ 5 primer

Bgl II site  
 5'- CCA **GAT CTC** CGC AAG TTG TAG GCT TGG TAC TC -3'

5' V $\delta$ 1 primer

Xho I site      Start Codon  
 5'- CCG **CTC GAG** CCA CCA **TGC** TTT GGA GAT GTC CAG TC -3'

3' V $\delta$ 1 primer

Bgl II site  
 5'- CCA **GAT CTC** CGC AAG GCT CTG AAA TTT GTG TG -3'

**Figure 2B. Thrombin linker region and fusion to  $\zeta$ .** Thrombin cleavage site is shown in bold and italics. Bgl II restriction site is shown as indicated. The cysteines involved in the interchain disulfide bonds in  $\gamma$ ,  $\delta$  or  $\zeta$  in bold. The terminal end of the 3' primers are shown to demonstrate their action in creating fusion of the  $\gamma$  or  $\delta$  gene to the thrombin linker region.

Bgl II site  
 V $\gamma$ 5      Thrombin linker region  
 ACT **TGC GGA GAT CTG GTT CCC CGG GGA TCA** TCA CGG CTG GAT CTC TGC  
TGA ACG CCT CTA GAC C      →  
 Terminal end of 3' primer      TCR $\zeta$

Bgl II site  
 V $\delta$ 1      Thrombin linker region  
 CCT **TGC GGA GAT CTG GTT CCC CGG GGA TCA** TCA CGG CTG GAT CTC TGC  
GGA ACG CCT CTA GAC C      →  
 Terminal end of 3' primer      TCR $\zeta$



### **c. pCDL-SR $\alpha$ 296 Vector**

The pCDL-SR $\alpha$ 296 vector was kindly provided by Carrie Steele, Ph.D. and Adrian Hayday, Ph.D. (Peter Gorer Department of Immunobiology of the Guy's King's St. Thomas' School of Medicine at Guy's Hospital, London, England). The construct encoded a TCRV $\delta$ 5 gene linked to a mouse TCR $\zeta$  gene and contained the SR $\alpha$  promoter from Simian virus 40 (SV40), which expresses in all cell types. The pCDL-SR $\alpha$ 296 vector contains a Pst I restriction enzyme site, which can be utilized to linearize constructs for transfection.

### **d. Digestion Reactions**

In order to remove TCRV $\delta$ 5, pCDL-SR $\alpha$ 296 was digested with Bgl II and Xho I enzymes. Reactions were performed in 20  $\mu$ l volumes, with 1 unit Bgl II (10,000 units/ml, New England BioLabs), 1 unit Xho I (20,000 units/ml, New England BioLabs), 1X BSA (100  $\mu$ g/ml; New England BioLabs), 1X Buffer 3 (50 mM Tris-HCl, 10 mM MgCl<sub>2</sub>, 100 mM NaCl, 1 mM 2,3-dihydroxy-1,4-dithiobutane {DTT}, pH 7.9 at 25°C; New England BioLabs), and 5-10  $\mu$ l pCDL-SR $\alpha$ 296, with the rest of volume made up with ddH<sub>2</sub>O. The digested pCDL-SR $\alpha$ 296 vector excluding the TCRV $\delta$ 5 gene was purified from 1% agarose 1X TAE gels using the protocol from the QIAEx®II Gel Extraction Kit (QIAGEN Inc.; Valencia, California, USA).

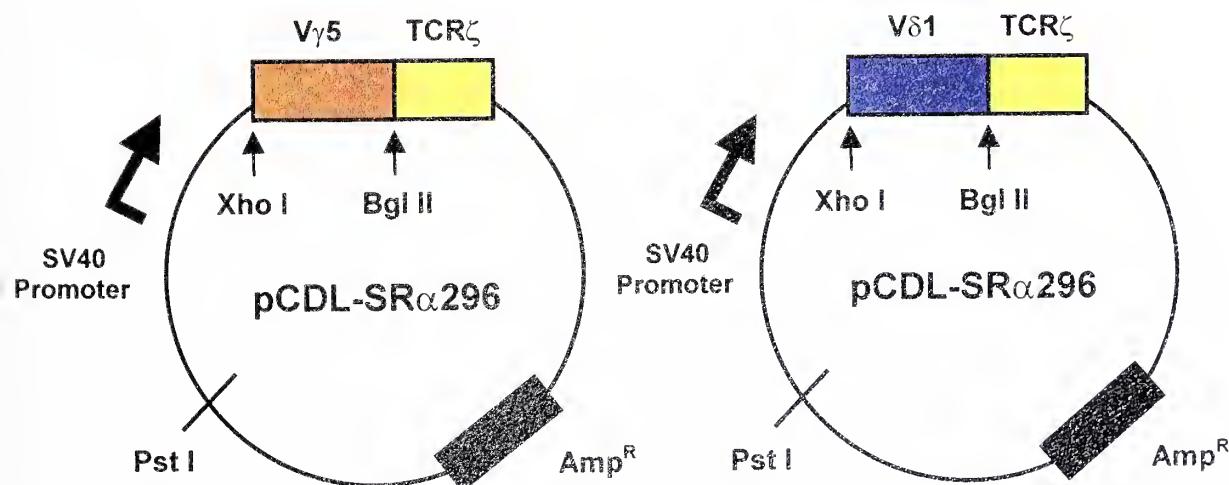
### **e. Ligation Reactions**

The V $\gamma$ 5 and V $\delta$ 1 cDNAs were cloned into the pCDL-SR $\alpha$ 296 vector already containing a thrombin linker and CD3 $\zeta$ , through the Xho I and Bgl II restriction sites for linkage. As a consequence, the extracellular domains of TCR $\gamma$  and  $\delta$  chains are fused



directly to the transmembrane and cytoplasmic domains of CD3 $\zeta$  (see Figure 3). Twenty- $\mu$ l ligation reactions were set up in 0.5 ml microcentrifuge Eppendorf tubes, consisting of 15.5-16.25  $\mu$ l of V $\gamma$ 5 or V $\delta$ 1 purified DNA, 0.75-1.5  $\mu$ l pCDL-SR $\alpha$ 296 vector, 1  $\mu$ l T4 DNA Ligase (400,000 units/ml, New England BioLabs), and 2  $\mu$ l 10X T4 Ligase buffer (New England BioLabs), such that the insert:vector concentration ratio was in the range of 10-20:1. Reactions were performed at 16°C for 2 hours, followed by heat-inactivation of T4 Ligase at 65°C for 10 minutes. Samples were placed on ice after reactions were completed.

**Figure 3. Completed constructs.** TCR V $\gamma$ 5 and V $\delta$ 1 chains were ligated into pCDL-SR $\alpha$ 296 using Xho I and Bgl II sites. pCDL-SR $\alpha$ 296 contains a Pst I site, which was used to linearize constructs for transfection. Amp<sup>R</sup> indicates the ampicillin resistance gene contained in the pCDL-SR $\alpha$ 296 vector.





### **f. Plasmid Transformations**

Plasmids were transformed into MAX Efficiency® DH5 $\alpha$ ™ Competent Cells (competent E-coli, Invitrogen; Carlsbad, California, USA). One, 4, or 15  $\mu$ l of ligation product were gently mixed into 50 ml of DH5 $\alpha$ ™ competent cells and incubated on ice for 30 minutes. The cells were then heat-shocked in a 42°C water bath for 45 seconds, followed by a 2-minute incubation on ice. Nine-hundred  $\mu$ l of SOC medium (2% Bacto®tryptone {Difco Laboratories, Detroit, Michigan, USA}, 0.5% Bacto®yeast extract {Difco}, 10mM NaCl, 2.5 mM KCl, 10mM MgCl<sub>2</sub>, 10 mM MgSO<sub>4</sub>, 20 mM Glucose) were added at room temperature. Vials were then shaken horizontally at 37°C for 1 hour at ~225 rpm.

### **g. Plasmid Isolation**

Newly-transformed DH5 $\alpha$ ™ competent cells were plated onto freshly prepared Luria-Bertani (LB) agar plates with 100  $\mu$ g/ml ampicillin using a flame-sterilized glass rod to spread cells in a circular motion until dried onto plate. Plates were placed lid-down in a plate incubator at 37°C overnight.

Five ml LB + 100  $\mu$ g/ml ampicillin cultures of transformed DH5 $\alpha$ ™ cells selected by individual colonies were grown in a horizontal shaker ~245 rpm at 37°C overnight. Bacteria were pelleted by centrifugation for 10 minutes at 2250 rpm (Sorval RT7plus). The supernatant was discarded, and the bacterial pellet was resuspended in 250  $\mu$ l of Buffer P1, provided in the QIAprep® Spin Miniprep Kit (QIAGEN Inc.; Valencia, California, USA), by repeated pipetting. Resuspended bacteria were transferred to a 1.5 ml microcentrifuge Eppendorf tube. DNA was isolated from bacteria as directed by the QIAprep® Spin Miniprep Kit manufacturers with the following



modifications. After the wash step with Buffer PE, samples in mini-columns were spun for 1 minute at 13,000 rpm. Samples were centrifuged for an additional 3 minutes to remove residual Buffer PE before proceeding to the elution step. At the elution step, 30  $\mu$ l of Buffer EB was added to the mini-column, followed by a 5-minute incubation at room temperature. Samples were then centrifuged at 13,000 rpm for 2 minutes. A second elution with 20  $\mu$ l of Buffer EB was then performed, with an additional 5-minute incubation at room temperature, followed by centrifugation at 13,000 rpm for 2 minutes. The eluates were combined in the same tube. These modifications were found to optimize DNA yield and concentration.

Digestion reactions with Xho I and Bgl II enzymes were set up for DNA obtained from each transformed colony to ensure that the proper V $\gamma$ 5 and V $\delta$ 1 chains were present in addition to the pCDL-SR $\alpha$ 296 vector. Single-enzyme digestions were set up as a control alongside double-enzyme digests. For double-enzyme digests, the reactions were performed in 20  $\mu$ l volumes, consisting of the following: 5  $\mu$ l of DNA, 2  $\mu$ l of Buffer 3 (New England BioLabs), 0.2  $\mu$ l 100X BSA (10 mg/ml; New England Biolabs), 0.5  $\mu$ l of Xho I (20,000 units/ml; New England BioLabs), 0.5  $\mu$ l of Bgl II (10,000 units/ml; New England BioLabs), and 11.8  $\mu$ l of ddH<sub>2</sub>O. Single-enzyme digests consisted of the following, also in 20  $\mu$ l total volumes: 5  $\mu$ l of DNA, 2  $\mu$ l of Buffer 2 (10 mM Tris-HCl, 10 mM MgCl<sub>2</sub>, 50 mM NaCl, 1 mM DTT, pH 7.9 at 25°C, New England BioLabs), 0.2  $\mu$ l of 100X BSA, 0.5  $\mu$ l of Xho I, and 12.3  $\mu$ l of ddH<sub>2</sub>O. Reactions were performed at 37°C for 2 hours. Digestion products were electrophoresed on 2% agarose 1X TAE gels, stained with ethidium bromide, and visualized under ultraviolet radiation.



To verify that correct sequences of the V $\gamma$ 5 and V $\delta$ 1 chains had been ligated into the pCDL-SR $\alpha$ 296 vector, samples of V $\gamma$ 5- and V $\delta$ 1-containing plasmid DNA (700 ng in 24  $\mu$ l volume reactions) were sent to the Keck DNA Sequencing Facility at Yale University for sequencing prior to their being used for transfection into heterologous cells.

## **2. Transfection into Rat Basophil Leukemia (RBL) Cells**

### **a. RBL-2H3 Cell Line**

The rat basophil leukemia cell line (RBL-2H3) was kindly provided by Carrie Steele, Ph.D., and Adrian Hayday, Ph.D. The RBLs were cultured at 37°C and 5% CO<sub>2</sub> in Minimum Essential Medium (MEM) with Earl's Salts (Gibco Life Technologies), 10% Fetal Calf Serum (FCS, Gibco Life Technologies), 1% Non-Essential Amino Acids (Gibco Life Technologies), 2 mM L-Glutamine, 100 units/ml Penicillin, and 100  $\mu$ g/ml Streptomycin. MEM with the stated added ingredients is referred to as complete MEM (CMEM).

### **b. Transfection Protocol**

For stable transfection, V $\gamma$ 5 pCDL-SR $\alpha$ 296 and V $\delta$ 1 pCDL-SR $\alpha$ 296 were linearized with Pst I (100,000 units/ml, New England BioLabs). Reaction concentrations were 40 units of Pst I, 38  $\mu$ g of plasmid constructs, 1X Buffer 3 (New England BioLabs), and rest of volume made up with ddH<sub>2</sub>O. To provide the neomycin resistance gene required for selection of transfectants, a plasmid called pCiNeo was linearized with Xho I (20,000 units/ml, New England BioLabs). Reaction concentrations were 25 units of Xho I, 21  $\mu$ g of pCiNeo, 1X BSA, 1X Buffer 2 (New England BioLabs), and rest of volume



made up with ddH<sub>2</sub>O. Linearized DNA was extracted with phenol:chloroform:isoamyl-alcohol (25:24:1, Merck), ethanol precipitated, and resuspended in 10-20  $\mu$ l of ddH<sub>2</sub>O, giving a final concentration of ~1  $\mu$ g/ $\mu$ l for each linearized construct.

The following steps were performed by Carrie Steele, Ph.D. at Guy's King's St. Thomas' School of Medicine Guy's Hospital in London, England. RBL-2H3 cells were trypsinized and resuspended in 0.25 ml of Dulbecco's Modified Essential Media (DMEM), with 16% FCS, 20 mM Hepes (Gibco Life Technologies), 2 mM L-Glutamine, 100 units/ml Penicillin, 100  $\mu$ g/ml Streptomycin, and 150  $\mu$ g/ml Gentamicin (Gibco Life Technologies) at a concentration of  $4 \times 10^7$  cells/ml. The cell suspension was transferred to a 0.4 cm gap cuvette with 9  $\mu$ g linearized V $\gamma$ 5 pCDL-SR $\alpha$ 296, 9  $\mu$ g V $\delta$ 1 pCDL-SR $\alpha$ 296, and 2  $\mu$ g pCiNeo. A charge of 260 V and 500 mF was applied with a gene pulser (Bio-Rad) at room temperature. Following a 10-minute recovery at room temperature, the cells were transferred back into CMEM for culture. Forty-eight hours after transfection, cells were selected in 0.5 mg/ml G418 sulfate (Gibco Life Technologies), with selection increasing to 1 mg/ml after another 48 hours. G418 is an aminoglycoside active against aerobic and facultative aerobic gram-negative bacilli as well as some gram-positive bacteria [230]. In general, this antibiotic is used for selection of transfecteds expressing an aminoglycoside-modifying enzyme, one of which is conferred to the RBL transfecteds used in this experiment by the co-transfection with pCiNeo containing the neomycin resistance gene. Control transfecteds were generated by transfection of RBL cells with pCiNeo alone to generate the RBLneo line.



### 3. Selection of Individual RBL $\gamma\delta$ Clones

Individual RBL $\gamma\delta$  clones were isolated in cloning wells through two methods. The first method entailed limiting dilutions, in which transfected RBL cells that had been selected by 1 mg/ml G418 were trypsinized and diluted to a concentration of ~1 cell/300  $\mu$ l. In a 96-well flat-bottomed plate, 100  $\mu$ l of cells at this concentration were transferred to each well and allowed to grow in CMEM at 37°C and 5% CO<sub>2</sub>. With such a strategy, individual clones would each have to be tested for the presence of the  $\gamma\delta$  TCR. The second method was utilized after a population of RBL $\gamma\delta$ + transfectants was identified by monoclonal antibody staining and flow cytometry (described below), from which RBL $\gamma\delta$ + cells were single-sorted into individual wells of a flat-bottomed 96-well plate. With this method, any clone that proliferated would be expected to express the  $\gamma\delta$  TCR.

#### a. Antibodies

The monoclonal antibodies (MoAb) to murine proteins used in this project were acquired from PharMingen unless otherwise stated. The following antibodies were used to stain RBL transfectants: phycoerythrin (PE)-conjugated hamster anti-C $\delta$  TCR (PE-GL3), fluorescein isothiocyanate (FITC)-conjugated hamster anti-C $\delta$  TCR (FITC-GL3), PE-conjugated anti-hamster IgG (isotype-matched control antibody), FITC-conjugated anti-hamster IgG (isotype-matched control antibody), rat IgM (control antibody), and FITC-conjugated anti-rat IgG + IgM (as secondary antibody to detect 17D1). 17D1 MoAb specific for DETC V $\gamma$ 5/V $\delta$ 1 TCR [64] was kindly provided by Lewis and Tigelaar (Yale University, Department of Dermatology).



### **b. Flow Cytometry**

Approximately  $1 \times 10^6$  cells were incubated with the appropriate MoAb on ice for 30-60 minutes, then washed twice in staining buffer (1X PBS/1% FCS) by centrifuging sample at 1200 rpm for 5 minutes, with staining buffer being discarded between washes. Cells were resuspended in 1 ml staining buffer and placed into Falcon® 2058 polystyrene tubes (Becton Dickinson; Sunnyvale, California, USA). Isotype-matched control antibodies were used at the same concentrations as test antibodies. Analysis was performed with a FACScan™ (Becton Dickinson) with electronic gates set on live cells by a combination of forward and side light scatter. A minimum of  $10^4$  live events was collected per sample and data were analyzed with CellQUEST™ software.

### **c. Fluorescence Activated Cell Sorting (FACS)**

RBL transfectants were incubated with PE-GL3 on ice for 30-60 minutes, then washed in staining buffer, all under sterile conditions. Cells were resuspended in 1 ml staining buffer and placed into sterile Falcon® 2058 polystyrene tubes. Transfectants were then analyzed by flow cytometry, and GL3 positive cells were subsequently sorted using Fluorescence Activated Cell Sorting (FACS) technology. FACS was performed with the generous help of staff at the Guy's King's St. Thomas' School of Medicine Guy's Hospital in London, England, using a Mo Flo high speed cell sorter (Cytomation). The V $\gamma$ 5/V $\delta$ 1+ RBL cells were cultured in 10% FCS/CMEM at 37°C and 5% CO<sub>2</sub>. This “enriched” population was allowed to proliferate until re-analysis with PE-GL3, FITC-GL3, and 17D1 with FITC-anti-rat IgG + IgM at a later time point determined by rate of proliferation. The percentage of V $\gamma$ 5/V $\delta$ 1 TCR+ RBL transfectants was expected to be significantly higher in any population in which such “enrichment” was performed.



#### 4. RBL-3H Serotonin Release Assay Protocol

The RBL transfectants were removed from culture flasks using Versene (EDTA), counted, and pelleted by centrifugation. The cells were then resuspended in 10% FCS/MEM + 1 mg/ml G418 media to a concentration of  $3 \times 10^4$  cell/100  $\mu$ l, and 100  $\mu$ l added to multiple wells of a flat-bottomed 96-well plate. Transfectants were loaded overnight at 37°C with 5-[1,2-<sup>3</sup>H(N)] hydroxytryptamine binoxalate (<sup>3</sup>H serotonin), at a concentration of 4  $\mu$ Ci/ml, or 0.4  $\mu$ Ci/well. The next day, cells were washed twice with ice-cold DMEM containing 2% FCS to remove excess serotonin that had not been incorporated into cells. Four testing conditions were set up in triplicate wells for an assay. First, GL3 hybridoma cells were used as effector cells in order to determine whether the transfectants would release serotonin in response to cell-bound ligand. In addition to secreting antibody specific for the constant (C) region of the  $\delta$  gene, the GL3 hybridoma expresses surface anti-TCR $\delta$  antibody which has been found to successfully cross-link the TCR on RBL $\gamma\delta$  transfectants, signaling for serotonin release in a dose-dependent fashion (Steele and Hayday, unpublished data). Second, transfectants were incubated with supernatant collected from cultures of 17D1 hybridoma cells, which contains soluble 17D1 antibody specific for the V $\gamma$ 5/V $\delta$ 1 TCR [64]. To determine if transfectants would release serotonin in response to as-yet unidentified ligands on keratinocytes, skin keratinocytes from the PDV line were used as target cells. Finally, phorbol myristate acetate (PMA)/ionomycin was used at a concentration of 0.5  $\mu$ g PMA +  $5 \times 10^{-5}$   $\mu$ moles of ionomycin per 50  $\mu$ L in each well. PMA/ionomycin causes a rise in intracellular Ca<sup>2+</sup> resulting in degranulation, thus allowing it to be an appropriate positive



control to demonstrate ability of RBL $\gamma\delta$  transfectants to release serotonin upon stimulation, albeit not through the  $\gamma\delta$  TCR.

Effector cells were prepared at a concentration of  $3 \times 10^5$  cells/well, i.e. a 1:10 RBL transfectant:effector cell ratio. Effector cells were reconstituted in a volume of 50  $\mu$ l/well, using 2% FCS/complete DMEM (DMEM with added 2 mM L-Glutamine, 100 units/ml Penicillin, and 100  $\mu$ g/ml Streptomycin). For the 17D1 supernatant, 50  $\mu$ l were added directly to the RBL cells. The plate was incubated for 45 minutes at 37°C. Supernatants were harvested and transferred to a UniFilter™ 96-well microplate with a built-in filter (PerkinElmer Life Sciences). Cells were then lysed in 1% Nonidet P-40 detergent in PBS (20mM Tris pH 8.0, 150 mM NaCl, 0.2% sodium azide, 1% NP-40 detergent), and lysates were transferred to the same UniFilter™ microplate in separate wells. The UniFilter™ microplate was incubated at 37°C until all wells were dried. To each well, 25  $\mu$ l of scintillation fluid was then added, and UniFilter™ plate was placed in a Microplate Scintillation & Luminescence Counter (Packard) to quantitate  $^3$ H content. Percentage  $^3$ H serotonin release was calculated using the following formula:

$$\% \text{ } ^3\text{H} \text{ serotonin release} = \frac{\text{counts per minute (cpm) in supernatant}}{\text{cpm in supernatant} + \text{cpm in lysate}}$$

Triplicate wells were averaged to obtain a mean %  $^3$ H serotonin release for each testing condition.



### III. RESULTS

#### A. TOTAL SERUM IgE MEASUREMENTS

##### 1. Lack of correlation between total IgE levels and SpD in C57BL/6 and NOD mice

Total serum IgE levels in wild-type (w.t.) and  $\delta$ –/– mice on C57BL/6 and NOD backgrounds were measured by ELISA. All B6 mice regardless of genotype were housed in CNVC. In contrast, groups of NOD w.t. and NOD. $\delta$ –/– mice were housed in different environmental settings, some in CNVC and others in IVC. As described in the Girardi et al. study (2002), the development of a spontaneous, chronic, localized dermatitis in  $\delta$ –/– mice, manifested by erythema, edema, and pruritus, along with a thickened epidermis showing histopathologic features typical of chronic dermatitis, was previously found to be strikingly genotype- and strain-dependent. Unmanipulated NOD. $\delta$ –/– mice developed a spontaneous cutaneous inflammation of the ears, while normal NOD controls did not. Additionally, both C57BL/6 w.t. and C57BL/6. $\delta$ –/– mice showed no clinical or histological evidence of spontaneous dermatitis. This SpD phenotype was further found to be environmentally-dependent, as NOD. $\delta$ –/– mice raised from birth in CNVC developed obvious dermatitis by 6 weeks of age, while age-matched NOD. $\delta$ –/– mice raised from birth in IVC failed to develop clinical or histologic evidence of dermatitis [231].

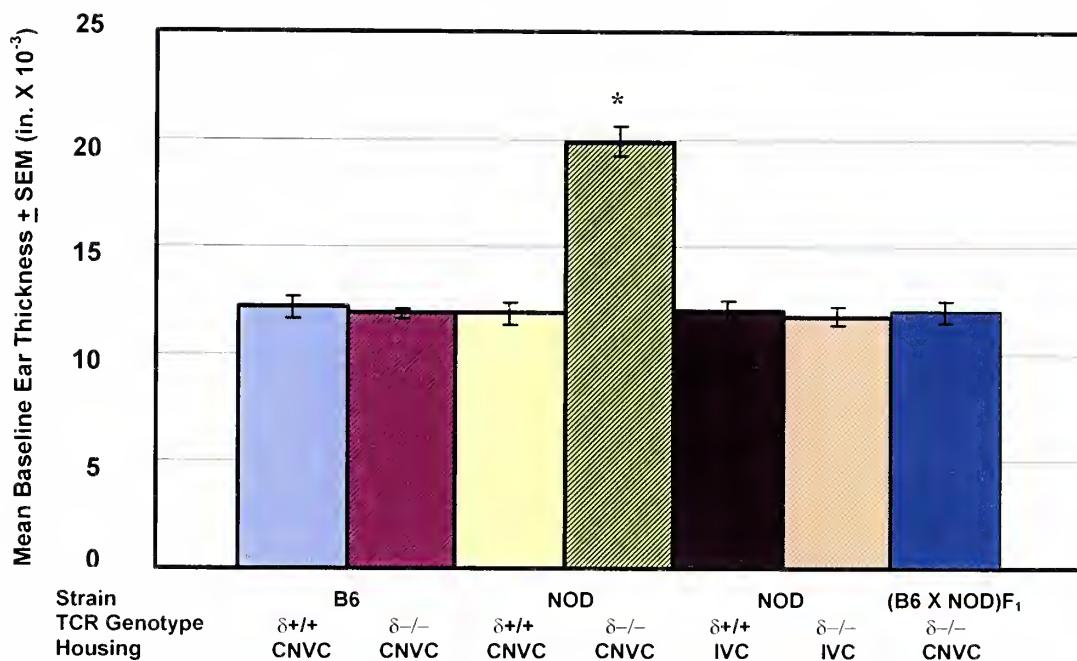
Based on these findings, with the hypothesis that IgE plays a critical role in the pathogenesis of this inflammatory dermatitis, it was expected that total serum IgE levels would correlate positively with the presence of SpD. In other words, NOD. $\delta$ –/– mice housed in CNVC, which presumably contains the appropriate environmental triggers



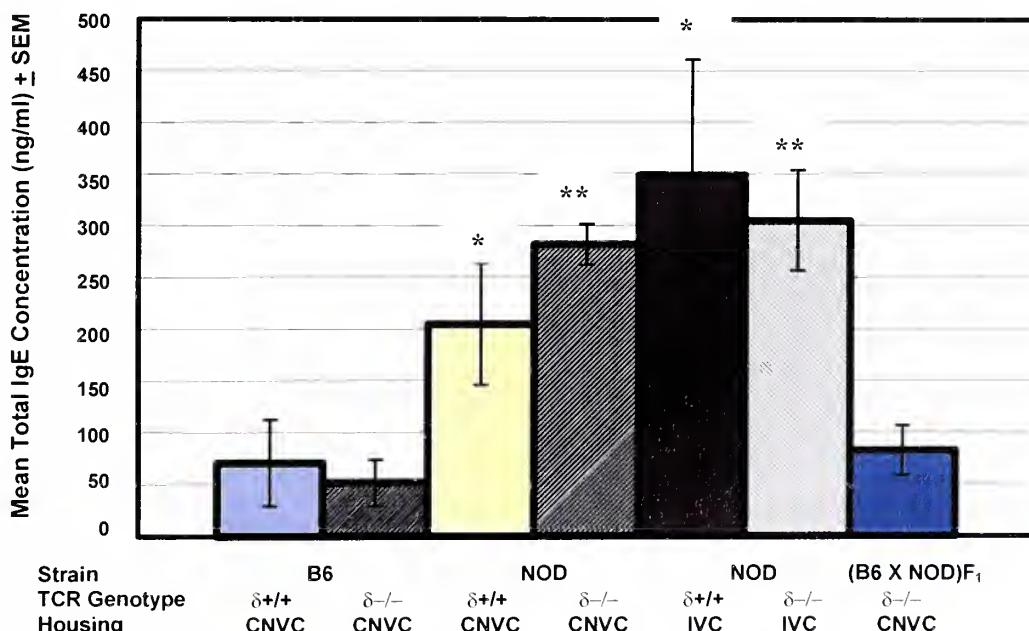
necessary to provoke an inflammatory process within the epidermis, would have significantly higher IgE levels than NOD. $\delta$  $-\text{}/-$  mice housed in IVC and NOD w.t. mice in either environment. In addition, it was expected that NOD. $\delta$  $-\text{}/-$  would show higher IgE levels than both B6 w.t. and B6. $\delta$  $-\text{}/-$  mice, which did not exhibit skin inflammation despite being housed with the same environmental triggers as the NOD. $\delta$  $-\text{}/-$  mice which showed inflammation.

As previously mentioned, ear phenotypes and baseline thicknesses of the specific animals (age 11-16 weeks) from which serum IgE levels were sampled were assessed on the day of serum collection. As expected, B6 w.t. mice housed in CNVC did not show any signs of cutaneous inflammation. In addition, B6. $\delta$  $-\text{}/-$  mice housed in CNVC did not develop signs of spontaneous dermatitis (SpD), having a mean baseline ear thickness of  $11.9 \pm 0.2$  in.  $\times 10^{-3}$  (Figure 4A). No signs of SpD were found in NOD w.t. mice housed in CNVC, which had a mean baseline ear thickness measurement of  $11.9 \pm 0.5$  in.  $\times 10^{-3}$ . Also as expected, NOD. $\delta$  $-\text{}/-$  mice housed in IVC had a comparable level of baseline ear thickness of  $11.8 \pm 0.4$  in.  $\times 10^{-3}$ . Meanwhile, NOD. $\delta$  $-\text{}/-$  mice housed in CNVC showed a baseline ear thickness of  $19.9 \pm 0.7$  in.  $\times 10^{-3}$ , significantly higher than NOD w.t. mice and NOD. $\delta$  $-\text{}/-$  mice housed in IVC ( $p < 10^{-7}$ ). These results were consistent with previous findings showing that such spontaneous, chronic cutaneous inflammation is dependent on several factors: 1) type of strain, developing in the NOD background and not the C57BL/6; 2) the presence of  $\gamma\delta$  T cells, developing only in  $\delta$  $-\text{}/-$  mice; and 3) the presence of appropriate environmental antigenic stimulation, developing only in mice housed in the CNVC environment [147, 231].





**Figure 4A.** Spontaneous dermatitis (SpD) developed only in NOD. $\delta^{-/-}$  mice housed in conventional non-ventilated cages (CNVC). None of the mice housed in individually-ventilated cages (IVC) showed signs of SpD. Of the mice housed in CNVC, only NOD. $\delta^{-/-}$  mice exhibited erythema and pruritus, as well as ear swelling manifested by an increase in baseline ear thickness. Ears were measured using an engineer's micrometer and are expressed as in.  $\times 10^{-3}$   $\pm$  standard error of the mean (SEM). \* $p < 10^{-7}$



**Figure 4B.** Serum total IgE levels and presence of SpD do not correlate. Total IgE levels (determined by ELISA) within a given strain did not differ based on the presence/absence of SpD or  $\gamma\delta$  cells, nor on the environment in which they were housed. IgE levels were significantly higher in both NOD w.t. and NOD. $\delta^{-/-}$  mice than in similarly housed B6 w.t. and B6. $\delta^{-/-}$  mice. \* $p < 0.05$  vs. C57BL/6 w.t. and C57BL/6. $\delta^{-/-}$ ; \*\* $p < 0.001$  vs. C57BL/6 w.t. and C57BL/6. $\delta^{-/-}$



Surprisingly, however, total serum IgE levels did not correlate with presence of SpD, nor with the environment in which they were housed. Total serum IgE levels of NOD. $\delta$ –/– mice housed in CNVC and those housed in IVC did not differ (Figure 4B). Furthermore, total serum IgE levels did not differ based on the presence or absence of  $\gamma\delta$  T cells. NOD w.t. mice, housed either in CNVC or in IVC, had levels of total serum IgE comparable to NOD. $\delta$ –/– mice (CNVC:  $204 \pm 59$  (SEM) ng/ml; IVC:  $349 \pm 112$ ). In contrast, total serum IgE levels were significantly strain-dependent. B6 w.t. mice housed in CNVC had an average total serum IgE level of  $70 \pm 42$  ng/ml ( $p < 0.05$  versus NOD w.t. housed in CNVC and in IVC), while B6. $\delta$ –/– mice had an average level of  $51 \pm 22$  ng/ml ( $p < 0.001$  versus NOD. $\delta$ –/– mice housed in CNVC and in IVC).

Among (B6 X NOD) $F_1$ . $\delta$ –/– mice housed in CNVC, no clinical signs of spontaneous dermatitis developed, and baseline ear thickness was indistinguishable from that seen in age- and sex-matched B6. $\delta$ –/– mice, consistent with results found by Girardi et al. (2002). Interestingly, the mean total serum IgE level found in (B6 X NOD) $F_1$ . $\delta$ –/– mice was also indistinguishable from that seen in B6. $\delta$ –/– mice, having a mean concentration of  $82 \pm 24$  ng/ml, suggesting that recessive gene(s) are responsible for the high total serum IgE levels in NOD (w.t. and  $\delta$ –/–) mice.

## **2. Lack of correlation between total IgE levels and SpD among ( $F_1$ X NOD)BC. $\delta$ –/– and (NOD X $F_1$ )BC. $\delta$ –/– mice**

Of 235 ( $F_1$  X NOD)BC. $\delta$ –/– and (NOD X  $F_1$ )BC. $\delta$ –/– mice, all housed in CNVC, 32 animals with the highest level of inflammation and 33 animals with the lowest level of inflammation were selected from which to obtain total serum IgE measurements. Individual ear measurements ranged from 11.00 to 28.50 in.  $\times 10^{-3}$ . The mean baseline



ear thickness of BC. $\delta$ –/– mice with high level of inflammation (“presence of SpD”) was  $22.5 \pm 2.7$  in.  $\times 10^{-3}$ , while that of BC. $\delta$ –/– mice with lack of inflammation (“absence of SpD”) was  $11.9 \pm 0.7$  in.  $\times 10^{-3}$  ( $p < 10^{-26}$ ) (Figure 5A). Again, contrary to the expected finding that animals with signs of SpD would show higher IgE levels than those which did not, no correlation was found between serum IgE concentration and the presence of SpD in BC. $\delta$ –/– mice. The mean total serum IgE level of BC. $\delta$ –/– mice with obvious inflammation was  $249 \pm 48$  ng/ml, while that of BC. $\delta$ –/– mice without inflammation was  $229 \pm 48$  ng/ml (Figure 5B).

These findings, therefore, do not lend support to the hypothesis that IgE antibodies play a primary role in the pathogenesis of SpD in genetically susceptible and appropriately housed mice. However, they do not formally exclude a role for IgE or other antibodies in the mechanism(s) accounting for the development of this phenotype.

### **3. Total IgE levels higher in females versus males**

In an analysis of BC. $\delta$ –/– mice segregated by sex, Girardi et al. (2002) had found that 48% (55/114) of females and 36% (44/121) of males were NOD-like. These differences between the male and female incidences of the susceptible phenotype were not statistically significant. Likewise, the frequency of phenotypically susceptible mice was similar in groups of BC animals segregated according to the sex of the NOD parent [147]. In sum, there was no statistically significant evidence for sex-linked genes affecting the development of spontaneous dermatitis. In this same study, it was found that baseline ear thickness in males tended to be higher than age-matched female counterparts. Such results are not surprising given the larger overall size of male animals



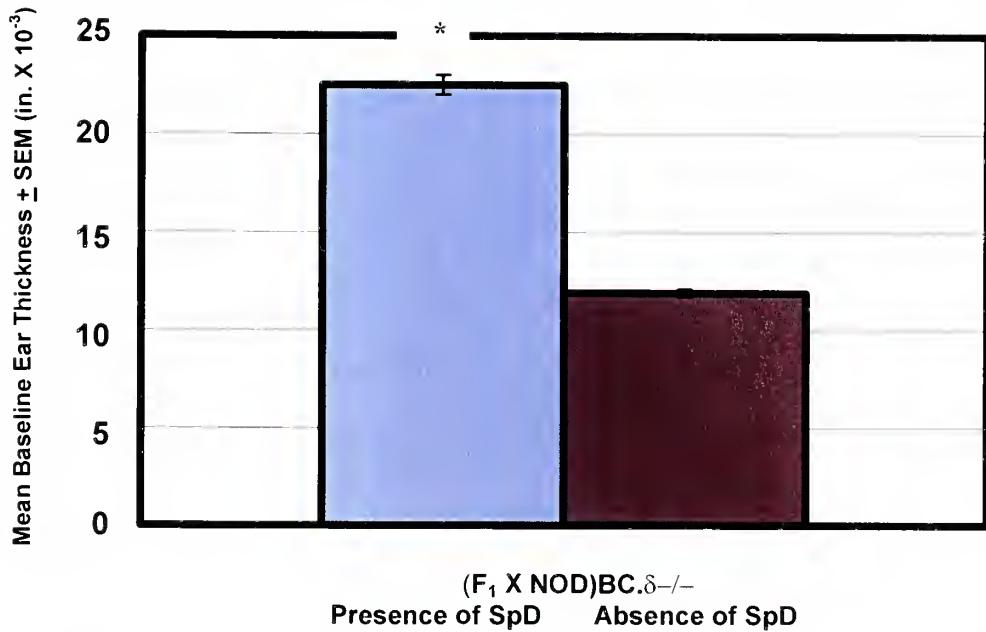


Figure 5A.  $(B6 \times NOD)F_1 \times NOD$  backcross  $(BC).\delta^{-/-}$  mice categorized as “presence of SpD” showed significant inflammation, as shown by significantly higher increased ear thickness measurements, compared to those with “absence of SpD.” Thirty-two (of 235) BC mice were identified to have the highest measurements of ear thickness (“presence of SpD”), while 33 BC mice were selected for their lowest ear thickness measurements (“absence of SpD”). The difference in mean baseline ear thicknesses was statistically significant.  $*p < 10^{-26}$

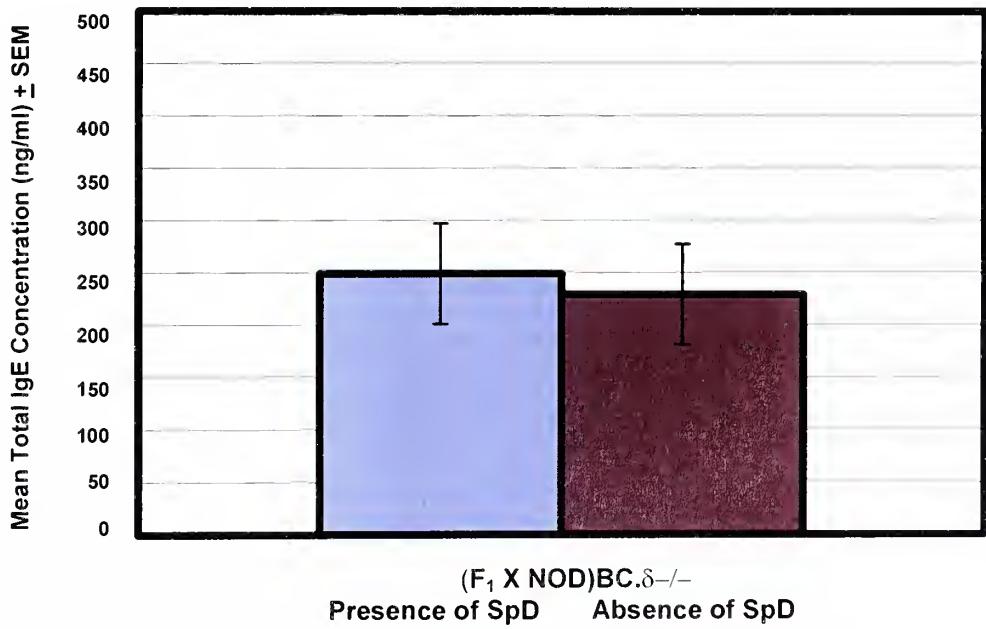


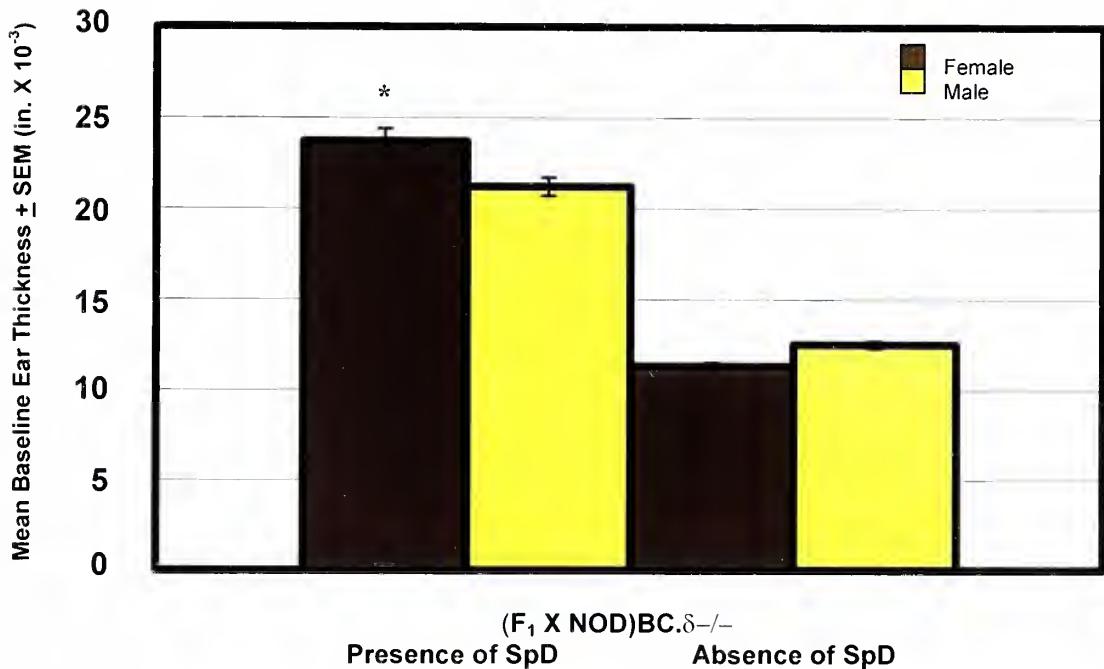
Figure 5B. The presence of SpD in  $(F_1 \times NOD)BC.\delta^{-/-}$  mice was not associated with higher IgE levels. Thirty-two (of 235) BC mice were identified to have the highest measurements of ear thickness (“presence of SpD”), while 33 BC mice were selected for their lowest ear thickness measurements (“absence of SpD”). There was no difference in IgE levels despite the difference in SpD development.



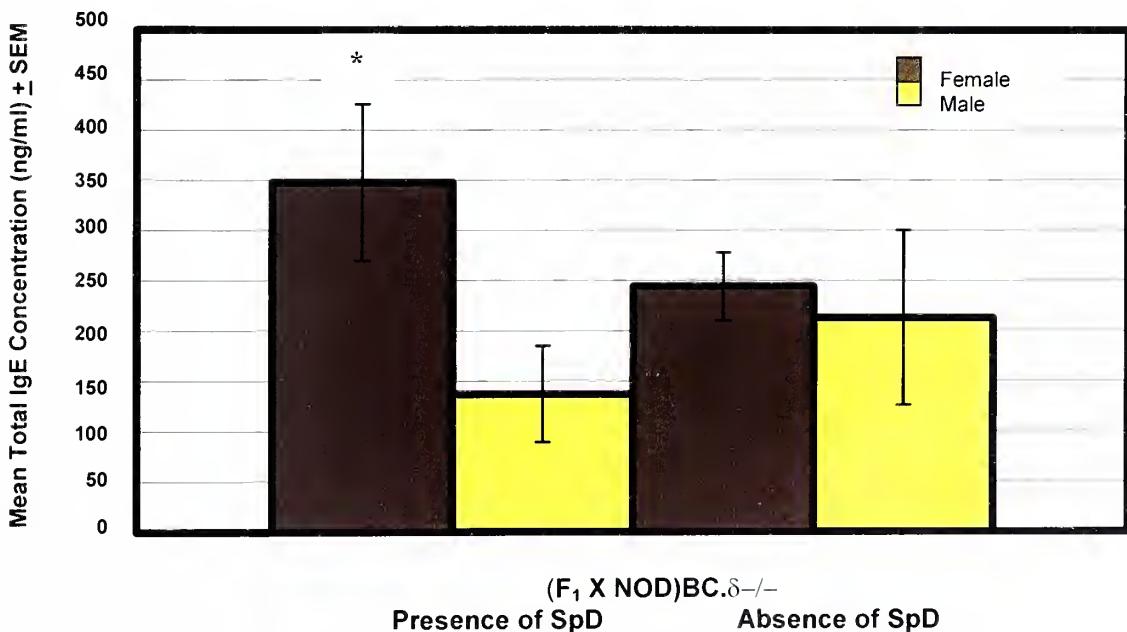
compared to females. In the present analysis, among the BC. $\delta$  $^{-/-}$  mice showing the lowest level of inflammation, measurements ranged from 11.00 to 11.75 in.  $\times 10^{-3}$  (mean=11.3  $\pm$  0.1) for females, and 12.25 to 13.00 (mean=12.5  $\pm$  0.1) for males ( $p < 10^{-13}$ ). Interestingly, however, among the BC. $\delta$  $^{-/-}$  mice with obvious SpD, female mice had a mean baseline ear thickness of 23.7  $\pm$  0.7 in.  $\times 10^{-3}$  (ranging from 20.25 to 28.50), while male mice had a mean ear thickness of 21.2  $\pm$  0.5 in.  $\times 10^{-3}$  (ranging from 16.75 to 24.00;  $p < 0.005$ ) (Figure 6A). More intriguing was the finding that the total serum IgE level in female BC. $\delta$  $^{-/-}$  mice with inflammation was significantly higher than that of males with inflammation (348  $\pm$  78 ng/ml vs. 137  $\pm$  34 ng/ml:  $p < 0.03$ ) (Figure 6B). Among BC. $\delta$  $^{-/-}$  mice which lacked signs of SpD, there was no difference in mean total serum IgE levels between males and females.

Female mice were also found to have higher IgE levels than their male counterparts in three other groups, all of which were mice deficient in  $\gamma\delta$  T cells and housed in CNVC (Figure 6C). In NOD. $\delta$  $^{-/-}$  mice housed in CNVC, which were the only animals showing evidence of spontaneous dermatitis, there was no difference in mean baseline ear thickness of females versus males. However, mean total IgE concentration was significantly higher in females compared to males ( $p < 0.05$ ). In B6. $\delta$  $^{-/-}$  and F<sub>1</sub>. $\delta$  $^{-/-}$  mice housed in CNVC, no animals developed spontaneous inflammation. Nonetheless, curiously, females compared to males were found to have significantly higher total IgE levels ( $p < 0.05$ ), whereas no significant difference in total serum IgE concentration was found between males and females among  $\delta^{+/+}$  mice or among  $\delta^{-/-}$  mice housed in IVC.



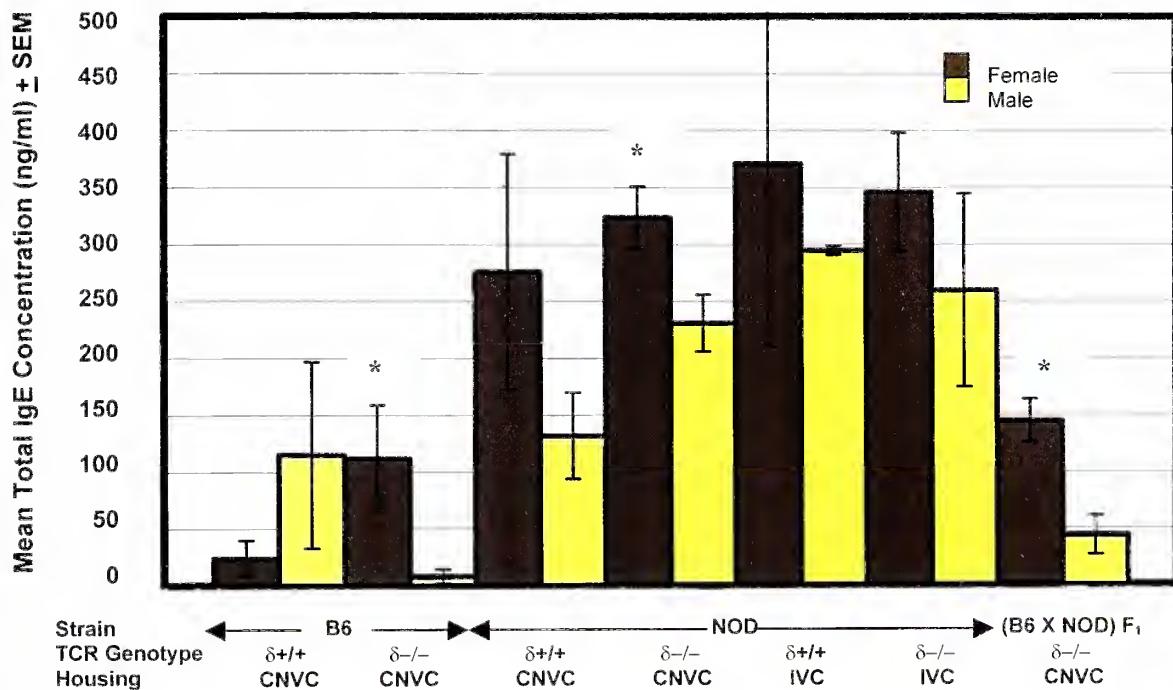


**Figure 6A.** Among BC.δ<sup>-/-</sup> mice showing SpD, female mice had a greater mean baseline ear thickness compared to male mice. Among BC.δ<sup>-/-</sup> without SpD, male mice had a greater mean baseline ear thickness compared to females, consistent with their larger overall size. In BC.δ<sup>-/-</sup> mice with SpD, on the other hand, female mice had a significantly higher mean baseline ear thickness than male mice. \* $p < 0.005$



**Figure 6B.** Among BC.δ<sup>-/-</sup> mice showing SpD, female mice had higher total serum IgE levels compared to male mice. This finding was in contrast from that seen among BC.δ<sup>-/-</sup> mice lacking signs of SpD, in which there was no difference in mean total serum IgE levels between males and females. \* $p < 0.03$





**Figure 6C. Female mice had higher total serum IgE levels than male mice.** In all groups of mice but one, female mice showed a tendency to have higher total IgE levels than their male counterparts. These differences were statistically significant only among  $\delta^{-/-}$  mice housed in CNVC.  $*p < 0.05$



## B. GENETIC ANALYSIS

### 1. Genetic interval(s) controlling susceptibility to SpD in NOD. $\delta$ –/– mice are distinct from those found for other mouse models of AD

Genome-wide microsatellite mapping studies in two other distinct mouse models of “atopic dermatitis-like” disease have recently mapped major susceptibility intervals controlling dermatitis to a region on chromosome 9 in NC/Nga mice and on chromosome 14 in NOA mice [152, 153]. With primers which distinguish NOD from B6 simple sequence length polymorphisms approximately in the centers of the previously described susceptibility intervals on chromosomes 9 (*D9Mit105*) and 14 (*D14Mit262*), analysis was performed of the DNA obtained from the 99 BC mice with obvious SpD using the criteria described in the previous chapter. If there were *no* linkage between the loci and the SpD phenotype, the expected number of samples exhibiting the NOD-specific SSLP on both chromosomes (NOD/NOD) would be exactly half the total number of samples tested, i.e. of 60 total samples, 30 would show NOD/NOD and 30 NOD/B6. Such a finding would lead to a  $\chi^2$  value of 0. A suggestive linkage, on the other hand, is defined by a  $\chi^2$  value of  $\geq 8.7$ , while significant linkage is defined by a  $\chi^2$  value of  $\geq 15.2$  [228]. The similar frequencies of homozygous (NOD/NOD) versus heterozygous (NOD/B6) genotypes for the *D9Mit105* and *D14Mit262* clearly indicate that the interval(s) controlling susceptibility to SpD in NOD. $\delta$ –/– mice are not the same as those found for the NC/Nga or NOA mice (Table 2). The genetic interval(s) controlling susceptibility to SpD in NOD. $\delta$ –/– mice are indeed distinct from intervals previously described for susceptibility to AD-like disease in other mouse models established to date.



	Chromosome 9 (D9Mit105)		Chromosome 14 (D14Mit262)	
Genotype	Observed	Expected	Observed	Expected
NOD/NOD	50	49.5	28	30
NOD/C57BL.6	49	49.5	32	30

**Table 2. The genetic interval(s) controlling susceptibility to SpD in NOD. $\delta$ –/– mice are distinct from those found for other mouse models of AD.**  $\chi^2$  analyses of the frequencies of homozygous (NOD/NOD) versus heterozygous (NOD/B6) genotypes for two specific genetic loci. Genome-wide microsatellite mapping studies in two other distinct mouse models of “atopic dermatitis-like” disease have recently mapped major susceptibility intervals controlling dermatitis to a region on chromosome 9 in NC/Nga mice and on chromosome 14 in NOA mice. With primers which distinguish NOD from B6 simple sequence length polymorphisms in the approximate centers of the previously described susceptibility intervals on chromosomes 9 and 14, analysis of the DNA (by PCR amplification and gel electrophoresis) obtained from the 99 BC. $\delta$ –/– mice with obvious SpD was done. The similar frequencies of homozygous versus heterozygous genotypes for these loci clearly indicate that the interval(s) controlling susceptibility to SpD in NOD. $\delta$ –/– mice are not the same as those found for the NC/Nga or NOA mice.



## C. CONSTRUCTION OF V5-J1-C $\gamma$ 1/V1-D2-J2-C $\delta$ T-CELL RECEPTOR

### 1. Generation of Expression Constructs

#### a. V $\gamma$ 5 and V $\delta$ 1 genes and chimeric constructs

V $\gamma$ 5 and V $\delta$ 1 cDNA were amplified from DETC cDNA produced by RT-PCR from freshly-isolated DETC mRNA. Primers were designed such that the PCR product would be flanked at its 5' end by an Xho I restriction site just before the leader sequence, and at its 3' end by a Bgl II restriction site. The Bgl II restriction site was added strategically to truncate the V $\gamma$ 5 and V $\delta$ 1 genes directly after the cysteine residues involved in the interchain disulfide bond. Such placement of the Bgl II site allowed for the direct fusion of the extracellular domains of V $\gamma$ 5 and V $\delta$ 1 to the thrombin linker region and CD3 $\zeta$ . TCR $\zeta$  provides the transmembrane domain for both chains. The V $\gamma$ 5 cDNA amplification showed the expected size of 779 bp, while the V $\delta$ 1 cDNA amplification showed the expected size of 767 bp. TCR $\gamma$  and TCR $\delta$  cDNAs were then purified from 1% agarose 1X TAE gels using QIAExII® gel extraction and gene sequences were confirmed by fluorescence based DNA sequence analysis on an ABI 377 automatic sequencer (performed on a fee-for-service basis in Yale University's Keck Center facilities).

The V $\gamma$ 5 sequence was found to have a conservative single point mutation at position 823, substituting a thymine base (T) for a cytosine base (C). The triplet sequences of CCT and CCC both code for proline, thus allowing for conservation of amino acid sequence. The V $\delta$ 1 sequence also showed a conservative single point mutation, this time at position 616, substituting a guanine base (G) for an adenine base (A). In this case, the triplet sequences of AAG and AAA both code for lysine, thus again allowing for conservation of amino acid sequence. Therefore, the sequences of both V $\gamma$ 5



and V $\delta$ 1 were confirmed to encode the proper sequence of amino acids to form the DETC TCR.

**b. pCDL-SR $\alpha$ 296 vector**

The pCDL-SR $\alpha$ 296 vector was kindly provided by Carrie Steele, Ph.D. from the laboratory of Adrian Hayday, Ph.D. (Guy's Hospital, London, England) as a construct containing a V $\delta$ 5 gene linked directly to a mouse TCR $\zeta$  gene through a region encoding a thrombin cleavage site. The V $\delta$ 5 gene was removed by digestion with the Xho I and Bgl II restriction enzymes, followed by purification of the digested pCDL-SR $\alpha$ 296 vector using QIAExII® gel extraction.

**c. Ligation Reactions**

The V $\gamma$ 5 and V $\delta$ 1 chains were ligated into the pCDL-SR $\alpha$ 296 vector through the Xho I and Bgl II restriction sites. The measured concentrations of the purified V $\gamma$ 5 and V $\delta$ 1 cDNAs were 4.69 ng/ $\mu$ l and 2.35 ng/ $\mu$ l, respectively. The concentration of the purified pCDL-SR $\alpha$ 296 vector was 14 ng/ $\mu$ l. In order to set up reactions with an insert:vector concentration ratio of 10-20:1, 15.5  $\mu$ l of V $\gamma$ 5 and 16.3  $\mu$ l of V $\delta$ 1 purified DNA were ligated into 1.5  $\mu$ l and 1  $\mu$ l of pCDL-SR $\alpha$ 296 vector, respectively. After the ligation reactions were completed at 16°C, samples were incubated at 65°C for 10 minutes to heat-inactivate the T4 ligase, followed by placement on ice and storage at -20°C until used for transformation.



#### **d. Plasmid Transformations and Isolation**

The complete plasmids were transformed into MAX Efficiency DH5 $\alpha$ <sup>TM</sup> Competent Cells and individual colonies were isolated and grown. Digestion reactions with Xho I and Bgl II enzymes were set up for DNA obtained from each transformed colony to ensure proper V $\gamma$ 5 and V $\delta$ 1 chains were present in addition to the pCDL-SR $\alpha$ 296 vector. Figure 7 shows single-enzyme and double-enzyme digests of two transformed colonies for V $\gamma$ 5-pCDL-SR $\alpha$ 296 and two transformed colonies for V $\delta$ 1-pCDL-SR $\alpha$ 296 (other colonies not shown). Each of these transformed colonies showed evidence of the appropriately-sized V $\gamma$ 5 and V $\delta$ 1 chain, along with the pCDL-SR $\alpha$ 296 vector, confirming successful transformation of the *E.coli* cells.

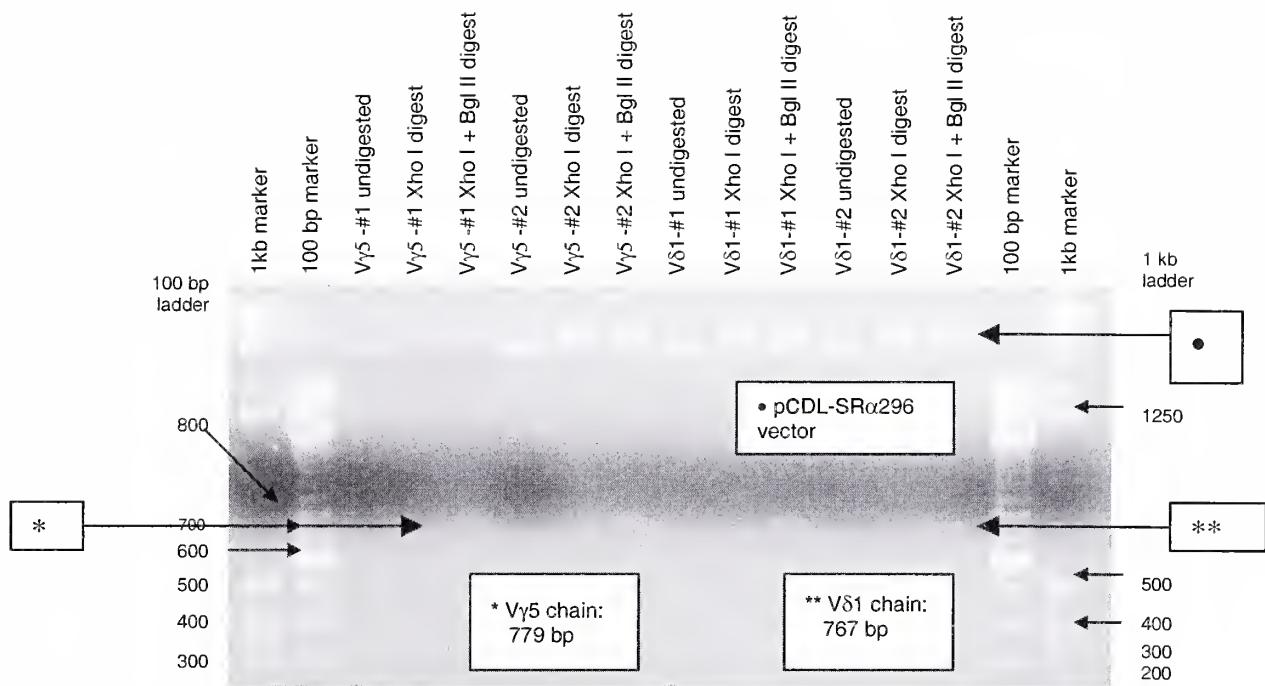
Approximately 700 ng of plasmid DNA from one colony transformed with the V $\gamma$ 5-pCDL-SR $\alpha$ 296 plasmid and 700 ng of plasmid DNA from one colony transformed with the V $\delta$ 1-pCDL-SR $\alpha$ 296 plasmid were sent for sequencing before being used in electroporation of the heterologous cell type RBL-2H3. Each of these colonies indeed showed the same sequence for the V $\gamma$ 5 and V $\delta$ 1 chains as those found for the chains before ligation into the vector and transformation of the *E.coli* cells (data not shown). Thus, conservation of correct sequences was confirmed.

## **2. Transfection of RBL-2H3**

### **a. Electroporation**

RBL-2H3 cells were transfected by electroporation with both V $\gamma$ 5-pCDL-SR $\alpha$ 296 and V $\delta$ 1-pCDL-SR $\alpha$ 296 constructs, linearized by digestion with Pst I, along with linearized pCiNeo. Cells were transferred back to CMEM for culture, then selected in





**Figure 7. Xho I single-enzyme and Xho I/Bgl II double-enzyme digests of *Vγ5*-pCDL-SR $\alpha$ 296 and *Vδ1*-pCDL-SR $\alpha$ 296 transformed colonies.** Each of the transformed *E.coli* colonies showed evidence of the appropriately-sized *Vγ5* or *Vδ1* chain and the digested pCDL-SR $\alpha$ 296 vector, confirming successful transformation of the bacteria with the *Vγ5*/*Vδ1*-pCDL-SR $\alpha$ 296 plasmids.



0.5 mg/ml G418 sulfate forty-eight hours after transfection, with selection increasing to 1 mg/ml after another 48 hours. The parent line of RBL-2H3 cells was also successfully transfected with pCiNeo only (RBL-Neo), allowing these cells to be used later as a negative control.

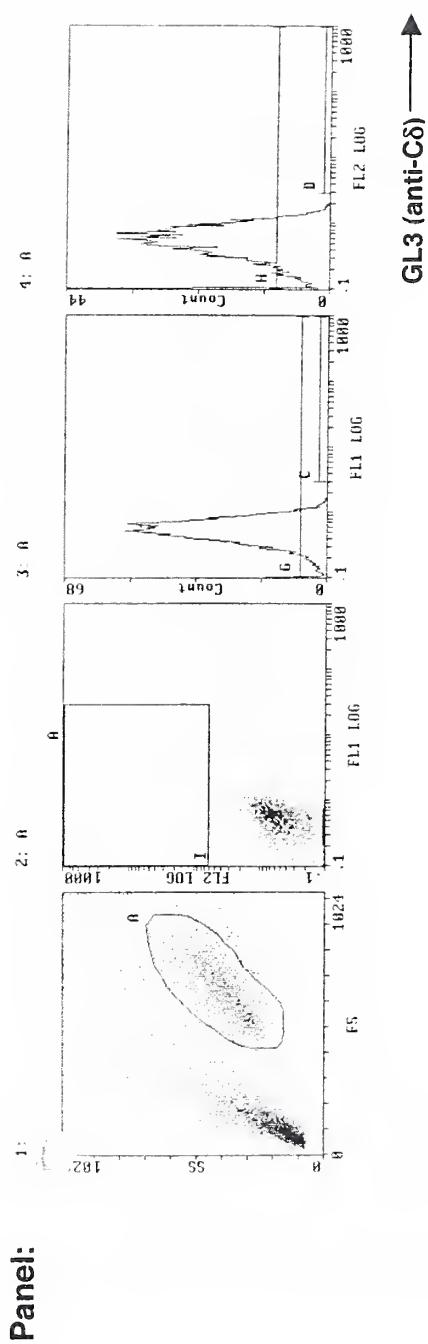
### **3. Transfectants Expressing V $\gamma$ 5/V $\delta$ 1**

#### **a. Enrichment of Bulk Transfectants**

Approximately  $4 \times 10^7$  RBL-2H3 cells were transfected simultaneously with V $\gamma$ 5-pCDL-SR $\alpha$ 296, V $\delta$ 1-pCDL-SR $\alpha$ 296, and pCiNeo plasmids. Selection with 0.5 mg/ml G418, followed by selection with 1.0 mg/ml G418, was carried out over 4 days. On post-transfection day 10 (PTD-10), the  $\gamma\delta$  transfectants that had been growing in a 10-ml petri dish were analyzed by flow cytometry, after staining the cells with PE-conjugated anti-C $\delta$  antibody (PE-GL3). Of the cells analyzed, 0.3% stained positive for GL3 (data not shown). As expected, C $\delta$  was not detected on RBL-Neo negative control transfectants (Figure 8A). Using FACS technology, 192 of the GL3-positive cells were sorted into 2 separate 96-well flat-bottomed plates, one cell per well, in order to develop clonal populations. Approximately 900 GL3-positive cells remained, which were then transferred in bulk to a 10-ml petri dish with normal culture media CMEM. Such “enrichment” was expected to show a significantly higher percentage of GL3-positive cells upon future analysis.

These “bulk” transfectants were allowed to grow in CMEM + 1.0 mg/ml G418 for an additional 10 days, at which point they were re-analyzed with PE-GL3. Approximately  $1.76 \times 10^6$  cells were counted, of which 10-12% were found to be GL3-





**Figure 8A. Flow cytometric analysis of RBL-Neo control transfectants.** TCR $\delta$  of the RBL-2H3 transfectants was stained with PE-GL3 (anti-C $\delta$ ). RBL-Neo control transfectants did not express a  $\gamma\delta$  TCR.



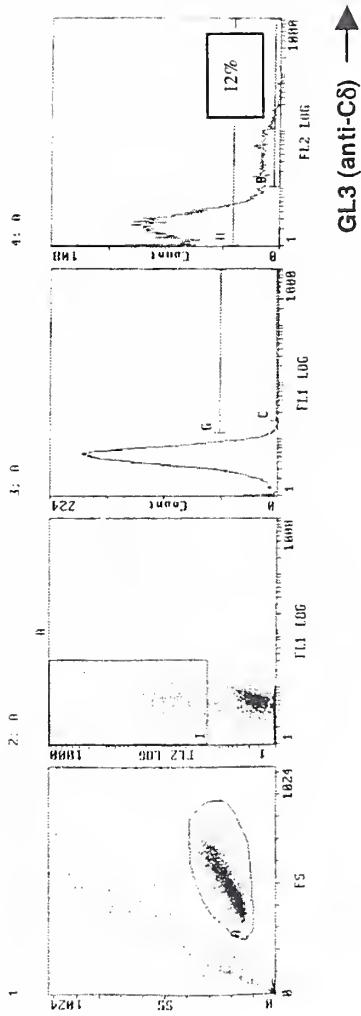
positive (Figure 8B). After analysis,  $1.2 \times 10^6$  cells were transferred into a medium-sized (T50 ml) flask to continue growing in culture.

Curiously, the RBL  $\gamma\delta$  transfectants at this point, after transfer to a 50-ml flask, did not show steady growth. In fact, a large proportion of cells quickly died, indicated by their floating in culture versus remaining attached to the bottom of the flask. The remaining cells that survived (exact percentage or number unknown) continued to appear attached to the flask bottom but did not exhibit visible proliferation. Discrete groups of cells began to grow in clumps, i.e. cells appearing to divide but accumulating on top of each other; however, the cultured cells did not reach confluence. Two weeks after having transferred the cells to a 50-ml flask (PTD 34), the RBL  $\gamma\delta$  transfectants were trypsinized and transferred to a flat-bottomed single well in a 6-well plate. Growth of the cells was soon visible, and on PTD 38, confluence of the cells in the well allowed for transfer of the cells to a 25-ml flask. On PTD 39, cells had reached confluence and were thus transferred to a larger 50-ml flask. Ten days later, on PTD 49, cells were re-analyzed with PE-conjugated GL3 antibody, at which point 18.5% of the cells were found to be GL3-positive (Figure 8C).

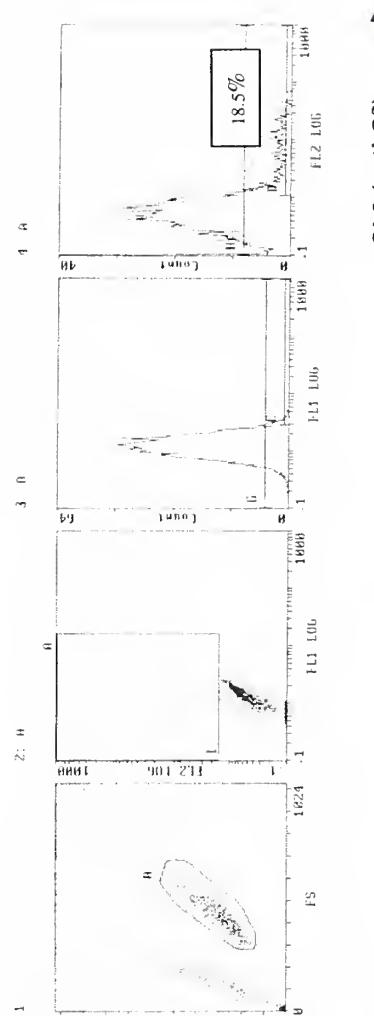
Cells were transferred to a 75-ml flask in order to allow proliferation, with the intention to freeze cells as stock for future sorting and culturing. Immediately prior to freezing, on PTD 59, FACS analyses showed that 16-17% of the RBL transfectants were GL3-positive, with a similar percentage (16%) of cells staining positively with 17D1 MoAb, detected by consequent staining with FITC-conjugated anti-rat IgG + IgM (Figure 9A and 9B).



**B)** Panel:



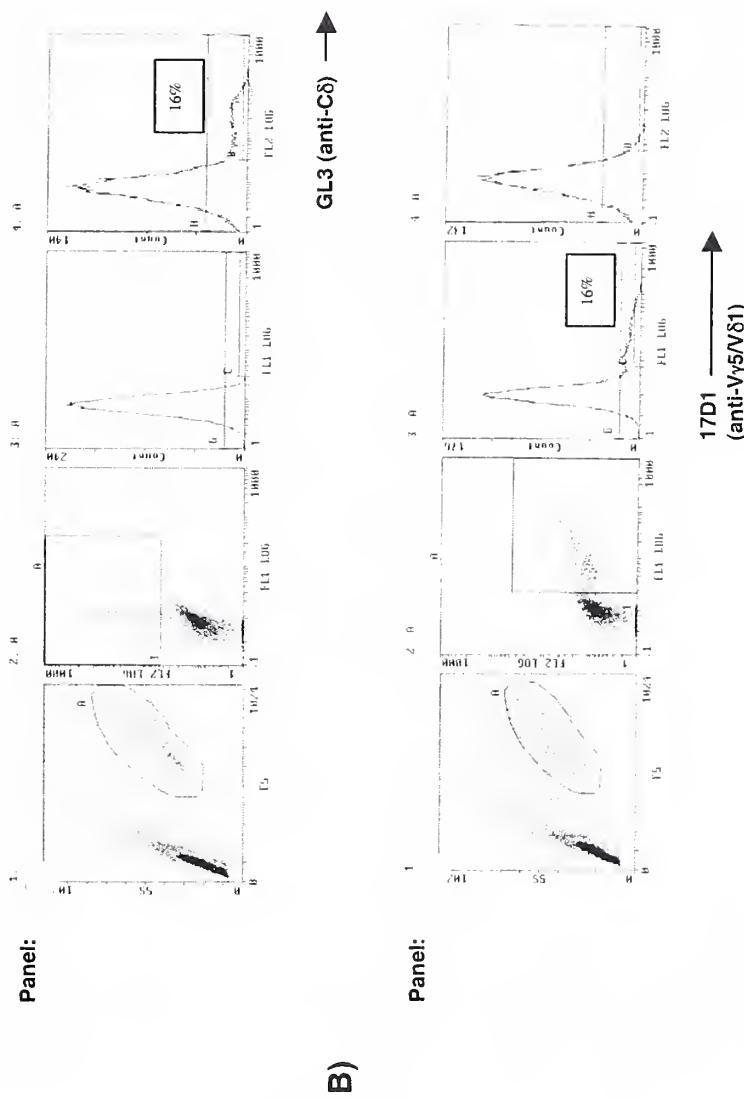
**C)**



**Figure 8B & C. Flow cytometric analysis of “enriched bulk” RBL V $\beta$ 5/V $\delta$ 1 transfectants.** B) Approximately 0.3% of the RBL-2H3 transfectants were initially PE-GL3 positive (data not shown). GL3-positive cells were further enriched by sorting using FACS technology. After 10 additional days of culture in selection (1.0 mg/ml G418) media, ~10-12% of RBL transfectants was found to be GL3-positive. Gate A in panel 1 indicates the population of cells analyzed. The other ungated population represents dead cells. Gate I in panel 2 surrounds the RBL cells staining positive for GL3. PE-GL3 fluoresces in the FL2 channel. Gate D in panel 4 indicates the RBL cells that fluoresce positively with PE-GL3, from which the percentage of cells positive for TCR C $\delta$  was determined. C) On the 49<sup>th</sup> day since the initial transfection (post-transfection day, or PTD, 49), this “enriched” population was re-analyzed and found to contain ~18.5% GL3-positive cells.



A)



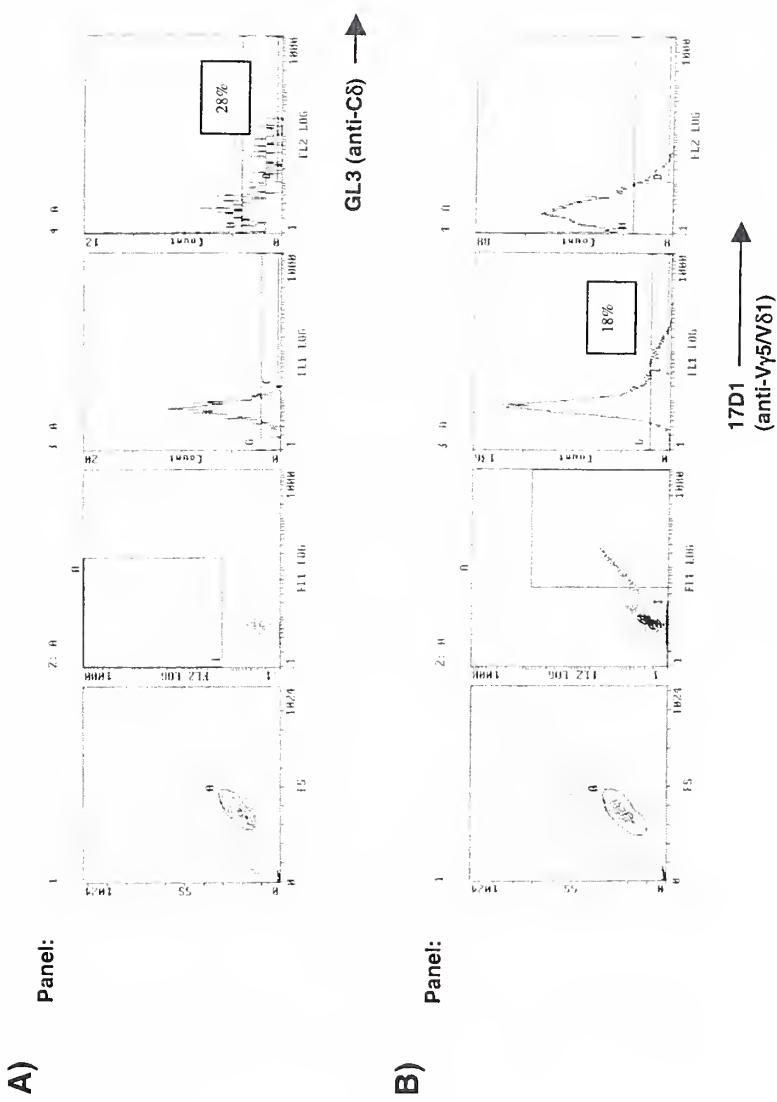
**Figure 9A & B. Flow cytometric analysis of “enriched bulk” RBL V $\gamma$ 5/V $\delta$ 1 transfectants on PTD 59.** A) TCR $\delta$  of the RBL-2H3 transfectants was stained with PE-GL3. By PTD 59, the “enriched bulk” transfectants showed ~16% to be GL3-positive, shown in Panels 2 and 4. B) Cells of this same population were stained for the V $\gamma$ 5/V $\delta$ 1 TCR (specific for DETC) with the MoAb 17D1 (anti-V $\gamma$ 5/V $\delta$ 1), then with FITC-conjugated anti-rat IgG + IgM. A similar percentage of ~16% was found to be 17D1-positive. FITC-conjugated 17D1 fluoresces in the FL1 channel, shown in Panel 2. Gate C in panel 3 indicates the RBL cells that fluoresce positively for 17D1, from which the percentage of cells positive for TCR V $\gamma$ 5/V $\delta$ 1 was determined.



## b. Clonal Populations

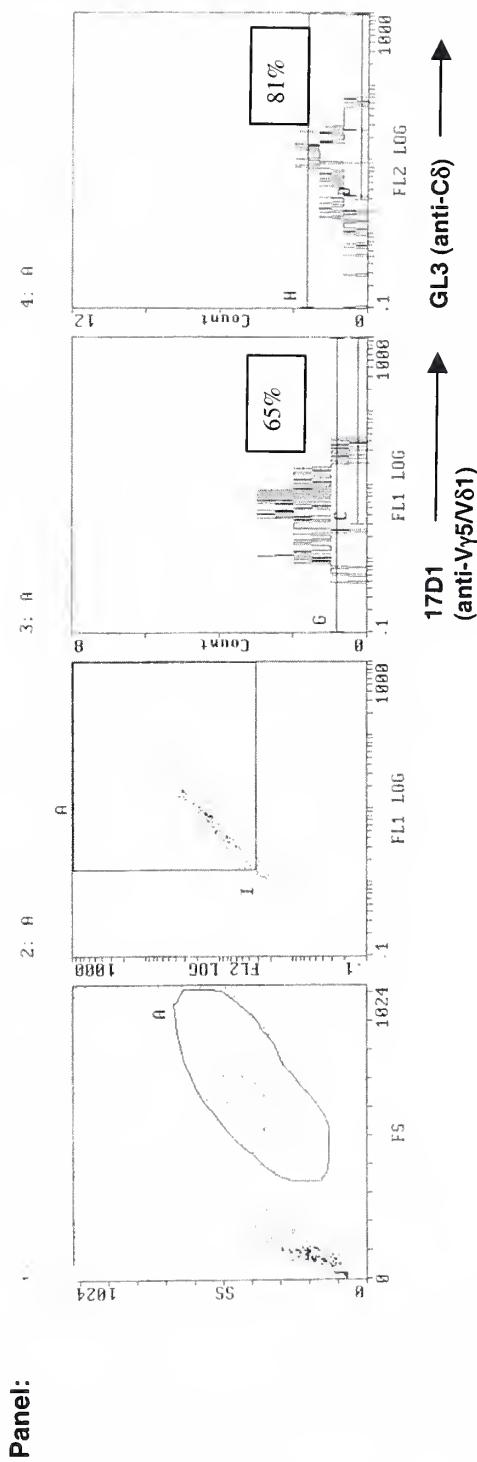
Of the 192 GL3-positive cells that were single-sorted into two 96-well plates, a total of approximately 23 clones on one 96-well plate and 20 clones on the other 96-well plate eventually grew into visible colonies. Approximately 50 of these colonies total were allowed to proliferate, being transferred only after they reached a minimum size (approximately 1 mm in diameter) and cultured into increasingly larger volumes of media. When colonies had expanded to approximately  $6 \times 10^5$  cells (reaching confluence in a single well of a 6-well plate), they were each analyzed by FACS to determine percentage of cells positive for GL3. Several colonies showed 0.1% GL3-positive cells, while others showed approximately 4% to 11%. Only one colony (#45) showed a substantially higher percentage of GL3-positive cells compared to the other colonies tested (Figure 10A). This colony was 28% GL3-positive when first analyzed 17 days after being picked from the 96-well plate (PTD 38). Staining with 17D1 MoAb on the cells of this colony on the same day showed the cells to be 18% positive, a slightly lower percentage than that found of GL3-positive cells (Figure 10B). On day 24 after being picked from the 96-well plate (PTD 45), clone #45 was analyzed again and the GL3-positive cells sorted by FACS. Of  $7.63 \times 10^6$  cells total, approximately 400,000 cells were GL3-positive. The GL3-positive cells were cultured for three days and then re-analyzed by double-staining, i.e. initial staining with PE-GL3, followed by incubation with 17D1 MoAb, and finally by staining with FITC-conjugated anti-rat IgG + IgM in order to detect 17D1. FACS analysis revealed cells to be 81% GL3-positive and 65% 17D1-positive (Figure 11).





**Figure 10A & B. Flow cytometric analysis of RBL V $\gamma$ 5/V $\delta$ 1 transfectant colony named Clone #45.** A) Of the 192 GL3-positive cells single-sorted from the original RBL transfectant population, ~50 colonies were chosen for proliferation. Several colonies showed 0.1% of cells to be GL3-positive, while others showed ~4-11%. One colony, Clone #45, analyzed on PTD 38, showed a markedly higher percentage of GL3-positive cells, ~28% (shown in Panels 2 and 4), compared to the other colonies. B) Clone #45 cells were also stained for the V $\gamma$ 5/V $\delta$ 1 TCR with 17D1, then with FITC-conjugated anti-rat IgG + IgM. A slighter smaller percentage of 18% was found to be 17D1-positive cells, shown in Panels 2 and 3.





**Figure 11. Flow cytometric analysis of “enriched” Clone #45 transfectants.** On PTD 45, Clone #45 cells were sorted by FACS into only GL3-positive cells. Staining of the TCR with both PE-GL3 and FITC-detected 17D1 three days later found cells to be 81% GL3-positive and 65% 17D1-positive.



#### 4. Serotonin Release Assay

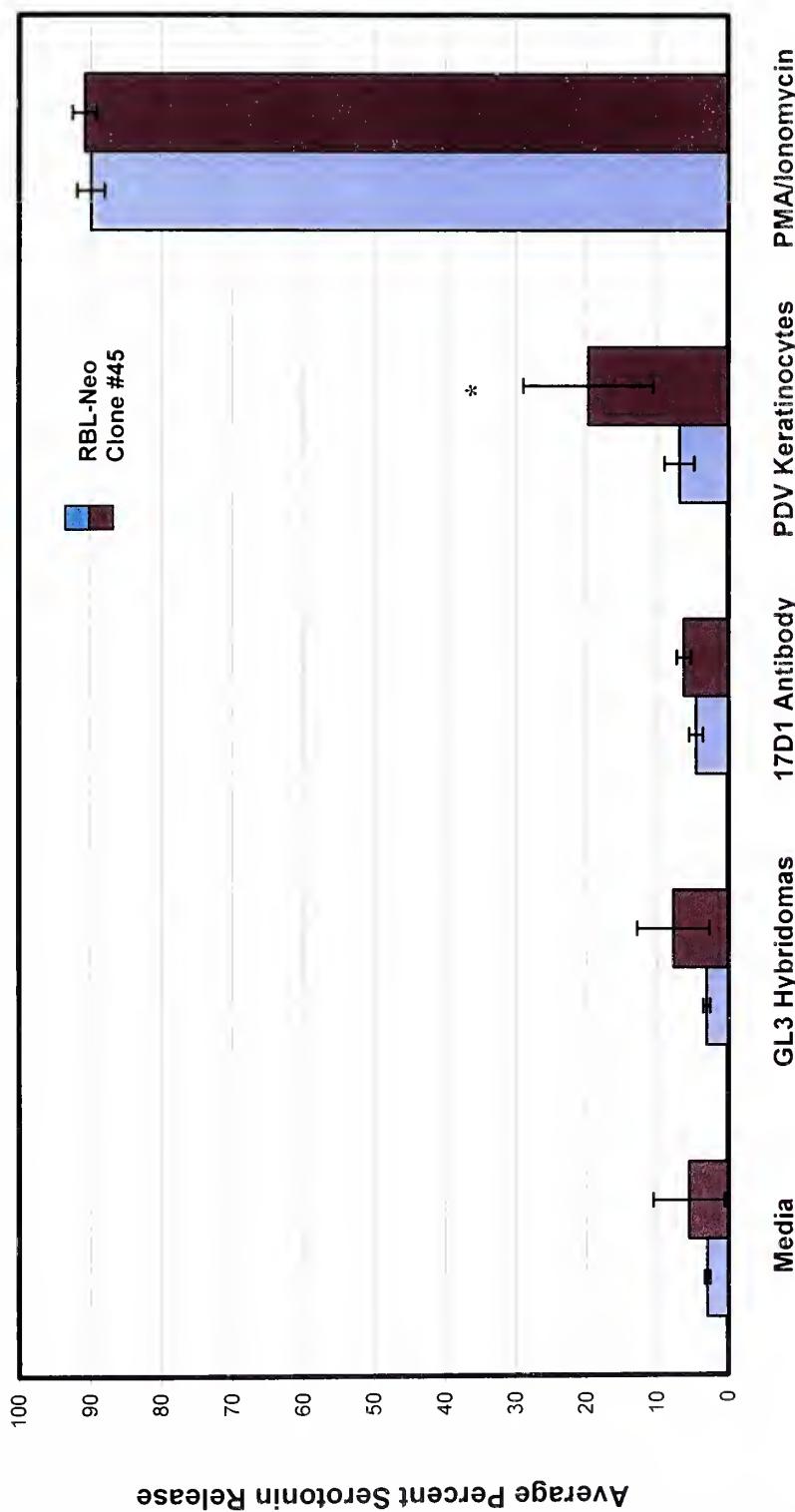
With the isolation and identification of a single RBL V $\gamma$ 5/V $\delta$ 1 TCR-positive clone, the salient question was to determine if the RBL transfectants would release serotonin in response to ligand. Clone #45 GL3-positive enriched cells (84% GL3-positive and 21% 17D1-positive) and RBL-Neo controls were cultured separately overnight in the presence of  $^3$ H serotonin. Activation was induced by incubating the plated cells in four different testing conditions. PMA/ionomycin was used to stimulate the RBL cells in order to show that the transfectants could indeed be activated to release pre-loaded  $^3$ H serotonin. PMA/ionomycin causes a rise in intracellular Ca $^{2+}$ , bypassing signal transduction and resulting in degranulation. Stimulation by PMA/ionomycin did indeed induce an 18-fold increase in serotonin release over control cells cultured only in media (Figure 12). There was no statistical difference in activation between Clone #45 and RBL-Neo control cells. Soluble 17D1 antibody specific for the V $\gamma$ 5/V $\delta$ 1 TCR was used to determine if the RBL transfectants could be activated by this monoclonal antibody targeting the specific TCR. Supernatant collected from 17D1 hybridoma cells was added directly to the RBL transfectants in a volume of 50  $\mu$ l. This sterile but unpurified supernatant containing 17D1 MoAb has been shown to successfully activate DETC lines (Lewis, personal communication), in which over 95% of cells express the identical V $\gamma$ 5/V $\delta$ 1 TCR [64]. In this assay, incubation of the RBL transfectants with 17D1 supernatant did not induce increased serotonin release compared to controls. To determine whether the transfectants would release serotonin in response to cell-bound ligand, GL3 hybridoma cells were used as target cells. The GL3 hybridoma expresses surface anti-TCR $\delta$  antibody, which cross-links TCR on the RBLs, leading to signaling for



serotonin release. GL3 hybridoma cells were incubated with RBL transfectants at a 1:10 RBL transfectant:effector cell ratio. In this assay, induced serotonin release was minimal, with no significant difference compared to controls. Activation by GL3 hybridoma in a dose-dependent fashion was not tested.

Lastly, an epithelial cell line was tested for expression of a ligand for the V $\gamma$ 5/V $\delta$ 1 TCR. Mouse PDV keratinocytes, which had been harvested from culture in a flask by scraping and were not heat-shocked or infected, were incubated with RBL transfectants. Upon activation, transfectants released preloaded serotonin at levels approximately 3-fold greater than that of RBL-Neo controls (see Figure 12), releasing up to 28% of preloaded  $^3$ H serotonin. Of the three replicate wells, there was one low outlying point which increased the standard deviation, making it fairly large (9%). Nonetheless, the percentage of serotonin release in response to activation by PDV keratinocytes was significantly greater for Clone #45 cells compared to RBL-Neo controls ( $p < 0.05$ ).





**Figure 12. Clone #45 RBL transfectants show induced serotonin release by PMA/ionomycin and PDV keratinocytes.** RBL-Neo and Clone #45 cells were plated at a concentration of  $3 \times 10^4$  cells/well in  $50 \mu\text{l}$  of 10% FCS/MEM containing  $4 \mu\text{Ci}/\text{ml} ^3\text{H}$  serotonin and cultured overnight at  $37^\circ\text{C}$ . Cells were washed twice with 2% FCS/DMEM, and then incubated for 45 minutes at  $37^\circ\text{C}$  with  $50 \mu\text{l}$  of effector cells in 2% FCS/DMEM, at a ratio of effector:transfectant cell of 1:4 for PDV keratinocytes and 1:10 for GL3 hybrids. PMA/ionomycin was used at a concentration of  $0.5 \mu\text{g}$  PMA +  $5 \times 10^{-5}$  mmoles of ionomycin per  $50 \mu\text{l}$ . Cell supernatants were harvested and counted. Cells were then lysed in 1% NP-40 in PBS, and the lysate content of  $^3\text{H}$  serotonin was measured. The percentage of released  $^3\text{H}$  was calculated. The percentage release shown is an average of triplicate wells  $\pm$  with 1 standard deviation.



## IV. DISCUSSION

### A. SPONTANEOUS DERMATITIS AND IgE

Atopic dermatitis (AD) is a common, chronic relapsing inflammatory skin disease, characterized by typically distributed eczematous skin lesions and intense pruritus, often associated with elevated levels of total and antigen-specific IgE and a personal or family history of atopic diseases [232-234]. Research into AD over the last 40 years has been dominated by exploration of the cellular and molecular mechanisms [151]. This work has indeed produced breakthroughs in elucidating the etiology and pathogenesis of this complex chronic disease. For example, skin lesions in AD consistently exhibit a mononuclear cell infiltrate, predominantly in the dermis, consisting of activated memory CD4+ T cells and macrophages [232], implicating a major role for both these types of cells in the pathogenesis of AD. Several findings indicate eosinophil involvement in AD, including the occasional presence of eosinophils in the dermis and the common finding of eosinophil-derived major basic protein and eosinophil cationic protein, which are associated with eosinophil degeneration [208, 235, 236]. Skin lesions in AD are also characterized by highly increased numbers of infiltrating, fully granulated mast cells [237], as well as increased numbers of dermal dendritic cells (CDs) and epidermal Langerhans cells [238, 239]. A reduction of ceramides in atopic skin, leading to a disrupted skin barrier has also been described as an etiologic factor [240]. Finally, the acute skin lesions of AD show a significant increase in the number of cells expressing the T<sub>H</sub>2 cytokines IL-4, IL-5, and IL-13 [241, 242], while in later stages of the disease, IL-12 expression increases and T<sub>H</sub>2 switches to T<sub>H</sub>1 immune responses, characterized by



the expression of such  $T_{H1}$ -type cytokines as IFN- $\gamma$  in chronic lesions [243]. Yet, despite these recent advances, the etiology and pathogenesis of AD are still not precisely understood. Various names have been assigned to this condition, including atopic eczema, neurodermatitis constitutionalis, endogenous eczema, eczema flexurarum, Besnier's prurigo, asthma eczema, and hay fever eczema, reflecting the uncertainty in the understanding of the exact pathophysiology of this disease [244, 245]. To further explore the pathogenesis and treatment of AD, suitable animal models are indeed required.

### ***SpD Similar to Human AD***

The spontaneous dermatitis (SpD) phenotype that developed in unmanipulated NOD. $\delta$ –/– mice of the present study has several similarities to features of human AD. The SpD was characterized by the clinical signs of erythema, edema, and pruritus, as well as the histopathologic features typical of chronic dermatitis, including a thickened epidermis with intercellular edema, mononuclear inflammatory cells scattered throughout the epidermis, and a thickened dermis with infiltrating mononuclear inflammatory cells, eosinophils, and highly increased numbers of mast cells [147]. The SpD shares other features with human AD as well, including: genetic predisposition [166, 183, 185, 246–248], appearing in  $\delta$ –/– mice on some (e.g. NOD and FVB), but not other (e.g. C57BL/6) genetic backgrounds; dependence on  $\alpha\beta$  T cells [249], appearing in  $\delta$ –/– mice but not in  $\beta$ –/– $\delta$ –/– mice; skin localization to areas commonly exposed to environmental irritants and/or allergens [168], developing only on the ear skin of these mice; and increased expression of contact dermatitis [250], showing strikingly augmented ear swelling to the allergen DNFB and the irritant TPA when compared to reactions induced in  $\delta$ +/+ mice.



Thus, the SpD induced in NOD. $\delta$ -/- mice housed in the appropriate environment (conventional non-ventilated cages) may provide a novel animal model for studying the pathogenesis of human AD.

### *The Role of IgE in Atopic Dermatitis*

The specific question addressed in the present study was the role of IgE antibodies in the pathogenesis of SpD. It is now widely accepted that IgE mediates immediate hypersensitivity, also called type I allergy [251]. The first step in an allergic reaction is the binding of a certain allergen to allergen-specific IgE molecules bound to the high-affinity IgE receptor (Fc $\epsilon$ RI) on mast cells, resulting in prompt release of a series of chemical mediators, such as histamine and serotonin, in addition to a variety of enzymes, including mast-cell chymase, tryptase, and serine esterases, which play important roles in initiating allergic-type and anaphylactic-type responses [165]. Upon activation, mast cells also secrete large amounts of cytokines, such as TNF- $\alpha$ , IL-4, and IL-13, chemokines, including MIP-1 $\alpha$ , which attracts monocytes, macrophages, and neutrophils, and lipid mediators such as leukotrienes and platelet-activating factor (PAF), all of which act in concert to orchestrate an inflammatory cascade that is amplified by the recruitment of eosinophils, basophils, and T<sub>H</sub>2 lymphocytes [252-254]. In contrast to the widely accepted role of IgE in immediate hypersensitivity, the pathologic role of IgE in chronic allergic inflammation as seen in atopic diseases, such as asthma and atopic dermatitis, is controversial [255]. It has been generally considered that chronic allergic inflammation is mediated mainly by T cells as opposed to IgE and mast cells [256]. Several lines of evidence point to an importance of T cells and other endogenous



cutaneous cells in AD. T cells comprise an important component of the histologic findings in chronic AD lesions, which feature the T cells in hyperplastic epidermis, often associated with GM-CSF production [257]. In the dermis, significant mononuclear cell aggregates are frequently observed in the perivascular space, reflecting the high degrees of trafficking of leukocytes, including macrophages, Langerhans' cells, dendritic cells, and lymphoid cells. Edema is another feature in the perivascular area of atopic skin, which is believed to be T-cell dependent [257]. Edema and pruritus in both acute and chronic lesions are associated with an abnormal pattern of epidermal infiltration by activated T cells and macrophages. Immunohistochemistry shows these T cells to be indeed activated, as demonstrated by HLA-DR expression [257]. Furthermore, empirically, therapies that inhibit T cells, through the suppression of T-cell activation, cytokine production, or migration, have been shown to ameliorate AD. Clinical studies using topical FK506 or tacrolimus, oral cyclosporine A, ASM 981, and photopheresis provide compelling evidence that T cells play a key role in the pathomechanism of AD [257].

If cell-mediated immune reactions are considered the predominant player in the pathomechanism of this disease, the atopic context manifested by increased IgE and IgE-mediated allergic reactions in the blood and skin of AD patients creates confusion. To further complicate the understanding of AD pathogenesis, high concentrations of total and allergen-specific IgE in the blood and skin are in fact not a consistent finding among AD patients. Two forms of AD have recently been proposed based on this discrepancy. The "allergic" or "extrinsic" form of AD (EAD) is associated with elevated levels of IgE and appears to comprise the majority of AD patients [258]. In the "nonallergic" or



“intrinsic” form (IAD), also known as nonallergic atopiform eczema or nonallergic atopiform dermatitis [259], which has been reported to represent 10-30% of all AD patients, patients have normal total serum IgE levels and no allergen-specific IgE, although they exhibit the same clinical phenotype [260]. This suggests that elevated allergen-specific IgE levels are not a prerequisite in the pathogenesis of IAD and key mechanisms contributing to this subtype of AD are still unknown. Conversely, however, recent studies have shown the ability of anti-IgE MoAb (omalizumab) to attenuate both immediate and delayed inflammatory responses, specifically in the treatment of allergic respiratory disorders [261, 262]. Such results suggest that IgE might indeed mediate allergic reactions beyond type I allergy. Though it is generally accepted that an immunological dysregulation of biphasic T<sub>H</sub>1/T<sub>H</sub>2 activity plays a central role in the pathogenesis of AD [263, 264], the specific role of IgE in the development of AD remains to be elucidated.

### ***Proposed Mouse Models of Human Atopic Dermatitis***

Total serum IgE levels found in mice of the present study did not correlate with presence of SpD, nor with the environment in which they were housed. While NOD.δ<sup>-/-</sup> mice housed in CNVC were the only group to exhibit signs of SpD, their total serum IgE levels did not differ from those of NOD.δ<sup>-/-</sup> housed in IVC or of NOD w.t. mice housed in either CNVC or IVC. Additionally, (F<sub>1</sub> X NOD) BC.δ<sup>-/-</sup> mice which showed obvious SpD did not demonstrate elevated total IgE levels compared to BC.δ<sup>-/-</sup> mice without SpD. These results do not support a primary role of IgE in the pathogenesis of SpD. If IgE antibodies played a predominant role in the development of SpD, one would expect



not only to see significantly elevated IgE levels in NOD. $\delta$ –/– mice housed in CNVC, which showed SpD, but also to see a direct correlation between IgE level and severity of clinical dermatitis, as measured, for example, by thickness of ear skin.

The findings from this study are also not consistent with those found in other mouse models of AD. For example, though similarities exist between the present findings and that of Matsuda et al. (1997) for the NC/Nga mouse model, one of the key differences is the finding of markedly increased total serum IgE levels in the latter. The atopic dermatitis-like lesions exhibited in NC/Nga mice are similar to those of the NOD. $\delta$ –/– mice in several ways. As in the present study, the development of spontaneous dermatitis occurred only in mice raised in conventional housing as opposed to a specific pathogen-free (SPF) environment consisting of a laminar filter-air flow enclosure in a bioclean room [198]. Clinical signs and symptoms seen in conventional NC/Nga mice were similar to those of NOD. $\delta$ –/–, albeit of a higher degree of severity and affecting areas not confined to the ears. The dermatitis was characterized by itching, erythema and hemorrhage, followed by edema, superficial erosion, deep excoriation, scaling and dryness of the skin, and alopecia in the face, ears, neck and dorsal skin, as well as growth retardation. Clinical severity of the dermatitis, examined in mice from 7 to 17 weeks of age, was minimal at 7 weeks old and steadily increased with age. Histological features of dorsal skin from conventional NC/Nga mice showed significant changes similar to those found in conventional-housed NOD. $\delta$ –/– mice and human AD lesions. These changes were apparent as early as at 7 weeks old and increased in degree with age, characterized by a thickened epidermis with moderate hyperplasia and prominent hyperkeratosis, intercellular edema, and increased numbers of mast cells and



eosinophils with degranulation [198]. Interestingly, Matsuda et al. (1997) also found that age-matched BALB/c mice maintained in the same conventional housing as NC/Nga mice from 7 to 17 weeks of age did not show any significant clinical signs or symptoms, similar to the findings that mice of the C57BL/6 background did not exhibit signs of SpD. In contrast to the NOD. $\delta$ -/- mice, conventional NC/Nga mice were found to have high levels of IgE (~25  $\mu$ g/ml) at 8 weeks of age, when mild dermatitis was clinically evident. By 10 weeks, IgE levels were markedly increased (~70  $\mu$ g/ml), and plateaued at 17 weeks, thus correlating with clinical severity of dermatitis. In contrast, plasma IgE levels in SPF NC/Nga and BALB/c mice remained at levels <0.5  $\mu$ g/ml. The direct correlation between IgE levels and clinical severity of dermatitis in conventional NC/Nga mice was similar to that reported in AD patients with IgE hyperproduction [265, 266].

The Naruto Research Institute Otsuka Atrichia (NOA) mouse is another strain that has served as a mouse model of human AD, as these mice develop severe ulcerative skin lesions that are associated with accumulation of mast cells and scratching behavior [193]. In NOA mice, high levels of serum IgE are found as well. NOA mice with ulcerative skin lesions show significantly higher serum IgE levels compared to NOA mice without skin lesions (~2,900 ng/ml vs. 400 ng/ml at 16 weeks of age; ~11,000 ng/ml vs. 450 ng/ml at 32 weeks of age), while control hairy mouse strains maintain very low levels of serum IgE (0-200 ng/ml) [193].

More recently, another mouse strain has been introduced as mice with naturally occurring dermatitis similar to human AD. DS-Nh originated from a mutant male mouse with deficient hair growth in an inbred DS strain colony at Aburahi Laboratories (Shionogi & Co., Ltd., Shiga, Japan) in 1976 [267]. The hairless phenotype of this



euthymic mouse shows an autosomal dominant inheritance, which is quite different from other well-known hairless phenotypes [268-270]. Heterozygous mutant DS Ng/+ mice housed under conventional conditions, assessed from ages 5 through 24 weeks old, were shown to develop skin symptoms similar to those of AD, including erythema, edema, excoriation, erosion, dry skin and desquamation of the forehead, lateral face, perioral area, neck, anterior chest and joint areas of their forelegs [267]. Early clinical symptoms, such as erythema, edema, excoriation, and dry skin, appeared in all mice at 9 weeks of age. Clinical severity increased steadily until the mice were 25 weeks old, at which time it remained stable. In contrast to conventionally housed DS-Nh mice, DS-Nh mice raised under SPF conditions, as well as conventionally housed DS mice, did not show any significant signs of spontaneous dermatitis. Notably, the authors found that NC/Nga mice housed in the conventional conditions used in their study failed to develop dermatitis over 30 weeks of observation. Significant histologic changes, including hyperkeratosis, acanthosis, slight intercellular edema of the epidermis, and infiltration of inflammatory cells, were observed in the skin lesions of all DS-Nh mice at 9 weeks or older, concurrent with the appearance of the clinical symptoms. Such histologic changes became more apparent with age. In facial skin tissue, conventional DS-Nh mice showed significantly higher numbers of degranulated mast cells and eosinophils compared to SPF DS-Nh and conventional DS mice [267]. Among DS-Nh mice raised under SPF conditions or in DS mice raised in SPF or conventional housing, no significant histopathological changes were observed throughout the 29-week observation period. Again in contrast to the findings in NOD. $\delta$ -/- mice of the present study, the level of total serum IgE was significantly increased only in conventionally housed DS-Nh mice and



not in DS-Nh mice raised under SPF conditions. Interestingly, serum IgE levels were not detected until 17 weeks of age (~200 ng/ml), but from that point on, levels subsequently increased with age (~2500 ng/ml  $\pm$  2000 at 20 weeks, ~4000 ng/ml  $\pm$  1200 at 25 weeks, and ~4500 ng/ml  $\pm$  1700 at 30 weeks).

### ***Comparison of NOD. $\delta$ –/– Mice with Other Mouse Models***

A few key observations can be made in comparing the findings from the present study with those from the previously proposed mouse models of AD. First, all the proposed mouse models, in addition to NOD. $\delta$ –/– mice, are similar in exhibiting a naturally occurring, spontaneous dermatitis, presenting AD-like symptoms and histopathological changes. Second, the spontaneous dermatitis occurs only in conventional conditions, as opposed to specific pathogen-free or ventilated housing conditions, implying the necessity of environmental triggers in initiating the chronic cutaneous inflammatory process. As described in these first two comparisons, the clinical and histopathologic features, as well as the dependence on environmental triggers, of the dermatitis exhibited in these mice all lend support to their validity in serving as mouse models for human AD. A third comparison between NC/Nga, NOA, and DS-Nh mice and NOD. $\delta$ –/– mice reveals an obvious difference between NOD. $\delta$ –/– mice and the first three mouse models. NOD. $\delta$ –/– mice do not exhibit elevated total serum IgE levels in association with the spontaneous dermatitis phenotype when compared to mice that do not exhibit the SpD phenotype. Though there was a lack of correlation between IgE levels and presence of dermatitis, a role for IgE in the



pathogenesis of SpD cannot be formally excluded in this study. The findings of this study can be interpreted in at least two ways.

### ***1. NOD. $\delta$ –/– as a Model for Atopiform Dermatitis***

One hypothesis explaining the lack of correlation between IgE levels and SpD is that the dermatitis observed in the present study is in fact not of an atopic nature. It is quite possible that the triggers for the SpD phenotype are as-yet unidentified factors found in the conventional non-ventilated cages that act on the skin as an irritant, and not an allergen. Because these mice do not demonstrate elevated levels of total serum IgE levels compared to controls, it is conceivable that the NOD. $\delta$ –/– mice may represent a novel model for “intrinsic” AD, or more accurately “atopiform dermatitis” [259]. It has been proposed that atopy should be defined as the genetically determined and environmentally influenced syndrome in which the primary immunological abnormality is the production of allergen-specific IgE [259]. Accordingly, the term “atopiform dermatitis” should be used to describe the condition, which affects 20% of patients with presumed AD, that is clinically identical to atopic dermatitis, but lacks the AD hallmark of allergen-specific and elevated levels of IgE. Recent evidence provides support for the hypothesis that patients with intrinsic AD display characteristics that differ from the phenomena typically seen in atopic disease. A hallmark of IAD is a lower Fc $\epsilon$ RI expression of CD1a+ epidermal dendritic cells [271]. Additionally, it has been shown that skin derived T cells from EAD give effective B cell help for IgE production, while T cells from IAD patients do not [212]. In a recent study on the phenotype of monocytes and serum levels of cytokines regulating the IgE production from nonatopic individuals



and patients with extrinsic and intrinsic dermatitis, the authors found the surface expression of the high- and low-affinity receptor for IgE (Fc $\epsilon$ RI and Fc $\epsilon$ RII/CD23) and the IL-4R $\alpha$  chain to be significantly elevated in monocytes from patients with EAD compared to those with IAD. Serum levels of IL-13 were significantly increased in patients with IAD, while levels of IL-5 were increased in patients with EAD. Furthermore, the frequency of the IL-4R $\alpha$  polymorphism C3223T and the IL-4 polymorphism C590T, which have been previously shown to play a relevant part in atopic diseases [272, 273], tended to be higher in EAD than in IAD. Thus, IAD patients appear to exhibit phenotypic and immunologic features distinct from those of patients with EAD or other atopic disorders [274], supporting the idea that these two forms of AD may have different pathogenic mechanisms.

If the SpD observed in the NOD. $\delta$ –/– housed in CNVC of the present study is in fact representative of an atopiform dermatitis, rather than a true atopic dermatitis, one may expect to see the phenotypic and immunologic features characterizing IAD patients in contrast to EAD patients. Indeed, further studies including serum measurements of cytokines such as IL-13 and IL-5 are feasible and warranted to further elucidate the pathogenesis of SpD. The characterization of Fc $\epsilon$ RI or Fc $\epsilon$ RII receptors on the monocytes and epidermal dendritic cells of the mice would also be useful in order to assess similarities or differences between this model and findings which characterize IAD. Intriguingly, however, studies to date have shown that murine epidermal Langerhans cells in fact do not express Fc $\epsilon$ RII at all [275], while the presence of Fc $\epsilon$ RI also has not been demonstrated. This finding suggests that murine Langerhans cells in



mice may hold a pathogenetic in spontaneous dermatitis distinct from that of their counterparts in human AD.

It is worth noting an interesting finding from a recently-established mouse line using the NC/Nga mouse model of AD. STAT6-deficient (STAT6<sup>-/-</sup>) NC mice were created in order to address the functional relevance of IgE and T<sub>H</sub>2-mediated immune responses in NC mice to the development of AD-like skin lesions [276]. STAT6 is a critical transcriptional factor that regulates IL-4-mediated immune responses. Phosphorylated and activated through an IL-4R-mediated signal, STAT6 translocates as a phosphorylated homodimer and subsequently regulates IL-4-mediated transcriptional events, including T<sub>H</sub>2 differentiation, expression of cell surface markers, and Ig class switching to IgE [277, 278]. STAT6<sup>-/-</sup> NC/Nga mice are unable to mount the T<sub>H</sub>2 cytokine production required for serum IgE response. Unexpectedly, these mice were observed to still elicit AD-like skin lesions at equivalent frequency and time of onset compared with normal NC/Nga littermates. Histological features of the lesions were consistent with the pathogenesis of AD, characterized by infiltration of eosinophils and mast cells. However, these mice failed to produce IgE and T<sub>H</sub>2 cytokines. The lymph nodes proximal to the skin regions with lesions showed massive enlargement elicited by the accumulation of activated IFN- $\gamma$ -secreting T cells. In addition, IL-18, IL-12, and IFN- $\gamma$  were found to be highly expressed at the skin lesions, along with elevated expression of eotaxin 2, a newly identified potent chemoattractant for eosinophils, and its receptor, CCR3. This study demonstrates that the T<sub>H</sub>2-mediated immune response is not necessary for the development of AD-like skin disease in NC/Nga mice. It is possible that the skin microenvironment, that of affected skin regions, which favors IFN- $\gamma$  and



IL-18 production and associated infiltration of eosinophils, may be more important than  $T_{H}2$  cytokines in the development of AD in NC/Nga mice [276]. This study lends further support to the idea that the SpD in NOD. $\delta$ –/– mice may not be atopic in nature, but have a mechanism that is independent of  $T_{H}2$  cells and IgE antibodies.

## ***2. NOD. $\delta$ –/– Mice as a Model for True Atopic Disease***

A second interpretation of the results from this study may be that the SpD in NOD. $\delta$ –/– is a form of true atopic disease, associated with elevated IgE levels, but the elevation was simply not detected at the time of serum collection. For example, in the DS-Nh mice discussed above, serum IgE levels did not become elevated until after 17 weeks of age, incongruent with the much earlier development of clinical symptoms, such as erythema and excoriation, at 9 weeks of age. The reason for an 8-week lag in IgE elevation is unclear. Yet based on this finding, it is indeed conceivable that if serum IgE levels are measured in the NOD mice serially starting from at least 5-6 weeks of age, when clinical symptoms first appear, for a longer time period, e.g. 30 weeks, a significant increase in IgE levels may in fact be detected, in a manner not necessarily coordinated with the appearance of clinical symptoms. This observation is in contrast to the finding in NC/Nga mice of the direct temporal correlation between IgE elevation and clinical development of dermatitis, and raises the question of the direct role of IgE in causing the AD-like lesions in these mice. Perhaps an additional factor driving the IgE synthesis is missing or blocked, even temporarily, and the pathogenesis of the disease is due primarily to non-IgE-mediated mechanisms, such as cytokines like IL-13 directly or indirectly driving the disease by the stimulation of eosinophils, interactions with B cells, or alterations of certain signal transduction pathways, like that of IL-13R, whose role has



previously been implicated in atopic diseases [279]. After the production of serum IgE begins to increase, IgE antibodies may begin to play a more direct role in the propagation of the disease. Mast cells, basophils, and eosinophils are certainly not the only cells of IgE-mediated action. Monocytes and dendritic cells such as Langerhans cells or the recently described inflammatory dendritic epidermal cells [280] have been shown to perform IgE-mediated antigen focusing, i.e. IgE increases their presenting capacity up to 100-1000 fold *in vitro* [281, 282]. Additionally, Fc $\epsilon$ RI has been found to be significantly upregulated on lesional epidermal dendritic cells of AD [280]. Three different IgE-binding structures have in fact been detected on epidermal antigen presenting dendritic cells; these include: (1) the low affinity IgE-receptor CD23/Fc $\epsilon$ RII [283]; (2) the high affinity IgE-receptor Fc $\epsilon$ RI [284, 285]; and (3) the IgE-binding protein Galectin 3/εBP [286]. The expression of such IgE-binding structures on these epidermal dendritic APCs emphasize the importance of these cells in the pathogenesis of IgE-mediated processes, even in chronic inflammatory diseases.

### ***Further Studies***

The study by Girardi et al. (2002) showed that not only NOD. $\delta$ –/– mice, but also FVB. $\delta$ –/– mice developed spontaneous inflammation of the ears as shown by highly significant but less dramatic differences in baseline ear thickness relative to age-matched FVB controls. The increased ear thickness was associated with qualitatively similar, albeit less intense, histologic differences as described for NOD. $\delta$ –/– mice. Interestingly, such changes developed in FVB. $\delta$ –/– mice in the absence of overt clinical signs of spontaneous dermatitis, i.e. pruritus or erythema [147]. It would be of interest to measure



serum levels of IgE in mice of the FVB background, and to compare measurements between FVB. $\delta$ –/– mice housed in CNVC, FVB. $\delta$ –/– housed in IVC, FVB w.t. housed in CNVC, and FVB w.t. housed in IVC, just as was done for NOD mice of the present study. It is curious that FVB. $\delta$ –/– mice exhibit histopathologic features of AD of lesser degree than NOD. $\delta$ –/– mice but of larger degree than C57BL/6. $\delta$ –/– mice, which developed neither clinical nor histologic evidence of spontaneous dermatitis compared to age-matched C57BL/6 controls. Perhaps serum IgE measurements would demonstrate levels that lie in between those found for NOD. $\delta$ –/– (average ~300 ng/ml) and C57BL/6. $\delta$ –/– mice (average ~50 ng/ml). Such a finding would suggest that perhaps mean baseline IgE levels are correlated with presence and/or severity of spontaneous dermatitis, despite not seeing a correlative increase in IgE levels with the development of SpD.

It is intriguing that among the proposed mouse models for AD, there is a markedly wide range in total serum IgE levels found among the different strains. In the NC/Nga mice, serum IgE levels were detectable starting at 6 weeks of age, as high as 3000 ng/ml. At 10 and 12 weeks of age, IgE levels were as high as 70 and 90  $\mu$ g/ml, or 70,000 and 90,000 ng/ml. Such levels of total IgE are almost 300 times greater than those found for NOD mice. Similarly, but to a lesser degree, serum IgE levels of DS-Nh mice were also reported to be significantly higher than those found in NOD mice, with averages of 2500, 3800, and 4500 ng/ml at 20, 25, and 30 weeks of age, respectively [267]. These levels are approximately 8 to 15 times higher than those found in NOD mice. In a transgenic mouse line expressing epidermal IL-4, established on the CByB6 strain and mated with non-transgenic BALB/cBy strain mice, IgE levels of affected mice



were found to be in the average range of 4000 to 5000 ng/ml, approximately 13 to 17 times higher than those of NOD mice [287]. Interestingly, in all these mouse models of AD-like dermatitis, the clinical signs and symptoms of the spontaneous, chronic inflammatory dermatitis were markedly more severe than those reported for the NOD. $\delta$ –/– and FVB. $\delta$ –/– mice. Conventionally-housed NC/Nga mice showed severe dermatitis with hemorrhage, edema, erosion, dryness and alopecia in the face, ears, neck and dorsal skin [198]. DS-Nh mice showed the early clinical symptoms of erythema, edema, excoriation, and dry skin, with increasing severity with age, associated with habitual scratching of their cheeks, eyelids, and neck [267]. In the IL-4 transgenic mice, skin lesions initially occurred at the mice’s ears and subsequently extended to the neck, mouth, around the eyes, tail, and legs [287]. There was severe dermatitis of the head and neck with areas of crusting, hair loss, and bacterial pyoderma as well as destruction of external ears. Eyelid dermatitis, blepharitis, and conjunctivitis resulted in corneal and conjunctival scarring, leading to blindness. In contrast, the spontaneous dermatitis exhibited in NOD. $\delta$ –/– mice housed in CNVC was very mild in comparison, consisting simply of pruritus, erythema, and edema, with visible lesions restricted to the ears. Indeed, the total IgE levels of ~300 ng/ml of these NOD mice are comparatively minuscule to those of the previously described mice. The comparisons described here support the idea that serum IgE levels are correlated with clinical severity of dermatitis, not only within a specific strain, but perhaps also across mouse strains. It is possible that genetic constraints exist which determine the potential of IgE production, even in response to appropriate environmental triggers. In turn, low levels of IgE production, i.e. a genetic “inability” or suppressive factor in producing high IgE responses to triggers,



would prevent the development of atopic dermatitis-like disease in the mice of such genetically-designed strains. Genetic studies are currently underway to elucidate such factors, as will be discussed in the next section.

Although the data of the present study do not appear to support a primary role of IgE in the pathogenesis of SpD in NOD. $\delta$ -/- mice, the requirement for immunoglobulins and/or B cells can be definitively determined by analyzing mice that are “double knockout” NOD. $\delta$ -/- Ig  $\mu$ -null mice [288]. If indeed such NOD. $\delta$ -/- Ig  $\mu$ -null mice which were exposed to the appropriate environmental triggers, i.e. CNVC housing, failed to exhibit signs of SpD, the necessity of immunoglobulins and/or B cells for the development of SpD would be revealed. On the other hand, if such mice continued to exhibit signs of spontaneous dermatitis, the conclusion could be made that immunoglobulins and/or B cells are not absolutely necessary for the initiation of this chronic cutaneous inflammatory process, and that other non-Ig-mediated processes are primarily responsible for the pathogenesis of this phenotype.

### ***Total IgE Levels Higher in Females versus Males***

In the present study, comparison of total serum IgE levels in female mice with those of male mice revealed an interesting observation. Among BC. $\delta$ -/- mice exhibiting obvious SpD, female mice compared to males showed a higher baseline ear thickness. Intriguingly, total serum IgE level in the females were also found to be higher than that of the males. When comparison of total serum IgE levels between females and males was done in all groups, significantly higher IgE levels were, in fact, found in three other groups of mice, all of which were  $\delta$ -/- mice housed in CNVC. Two of these groups



were B6. $\delta$ –/– mice and (B6 X NOD) F<sub>1</sub>. $\delta$ –/– mice, which did not even exhibit signs of SpD. The explanation for this finding is unclear, but readily suggests a possible female-specific factor that is contributing to increased IgE responsiveness to the environmental trigger(s) causing SpD. A review of literature did not reveal previous reports of higher total IgE levels in female AD patients compared to males, or in female mice compared to males in other mouse models of AD. However, there are several reports of slightly increased prevalence of AD in females, with a male to female ratio of 1:1.5 [219, 289]. Additionally, several studies have reported maternal atopy to cause a greater risk of atopic disorders in the offspring than paternal atopy [219, 288, 290-296]. Though the reasons for this phenomenon is still unclear, genetic studies have shown specifically maternal patterns of transmission at certain loci associated with AD. For example, Cookson et al. (1992) found significant sharing of maternal alleles of chromosome 11q13, on which a gene for atopy has been assigned [297], in sibling-pairs with atopic IgE responsiveness (defined by higher than normal total serum IgE levels) [175]. The excess sharing of maternal alleles occurred whether or not the mother was herself overtly atopic, which points to the possibility of genomic imprinting. It was noted that human 11q13 is syntenic to mouse chromosome 7F1-ter, in one of only seven areas recognized to show genomic imprinting [298]. Such linkage of atopic IgE response to chromosome 11q13 maternally derived alleles was found in a separate study of Japanese families, providing support for a maternal inheritance pattern [299]. A specific examination of gene polymorphisms for the  $\beta$  chain of the high affinity receptor for IgE (Fc $\epsilon$ RI- $\beta$ ), which is a candidate accounting for the chromosome 11q13 effect on atopy, found that all 13 children in the study who had inherited the polymorphisms Leu181/Leu183



maternally were atopic. Leu181/Leu183 had previously been found to be associated with atopy [299]. By contrast, the eight children who had derived the variant paternally had negative skin prick and radioallergosorbent test results and did not have increased bronchial responsiveness. Thus, the authors concluded that these polymorphisms of Fc $\epsilon$ RI- $\beta$  identified a genetic risk factor for atopy and bronchial hyperresponsiveness when inherited maternally [300]. More recently, two more polymorphisms within the Fc $\epsilon$ RI- $\beta$  gene, namely allele 2 of *Rsa*I intro 2 and allele 1 of *Rsa*I exon 7, were found to be strongly associated with AD [301]. Furthermore, transmission of these alleles was significantly associated with AD when maternally-inherited. Though these studies do not directly parallel the observation of higher total serum IgE levels in females over males in the present study, they do lend support to a female bias in both atopy prevalence and atopic IgE responsiveness. Further studies are warranted both to confirm these findings among the B6. $\delta$ -/- and NOD. $\delta$ -/- mice and to elucidate the mechanism by which this is occurring. Perhaps, for example, the females in these mouse strains possess a gene that codes for increased expression of the high-affinity IgE receptor, Fc $\epsilon$ RI, which would lead to increased IgE responsiveness to environmental allergens. Examination of genes associated with atopy or AD may in fact reveal differences in polymorphisms between females and males.

### ***Role of DETC in Atopic Dermatitis***

From the observation that NOD. $\delta$ -/- mice developed spontaneous signs of SpD, while NOD. $\delta$ +/+ mice housed in the same CNVC conditions did not, it is apparent that the deficiency of  $\gamma\delta$  T cells, presumably DETC, contributed to the pathogenesis of SpD.



In fact, Girardi et al. (2002) showed through reconstitution of  $\delta$ -/- mice specifically with V $\gamma$ 5+ fetal thymocytes (DETC precursors) that V $\gamma$ 5+, but not V $\gamma$ 5-, cells had the ability to down-regulate both spontaneous and irritant dermatitis in the  $\delta$ -/- mice. These results provide a novel demonstration that a local functional impairment at an epithelial interface can be attributed to the specific absence of the local  $\gamma\delta$  cell subset constitutively resident within that tissue, and that DETC provide local, nonredundant regulation of cutaneous inflammation [147]. In considering the NOD. $\delta$ -/- mice as a possible mouse model for AD, the question is raised of the role of DETC specifically in allergic disease. In a study of Brown Norway rats, tolerized to OVA by repeated aerosol exposure [302],  $\gamma\delta$  T cells were found to produce high levels of IFN- $\gamma$  in response to ovalbumin (OVA) stimulation *in vitro*, suggesting a mechanism for the inhibition of Th2-type antigen responses, and therefore for the selective suppression of IgE antibody production.  $\gamma\delta$  T cells also exhibited the unique potential of selective IgE antibody suppression in response to mucosal antigen exposure; thus, it was further proposed that  $\gamma\delta$  T cells may play an important role in protection against primary allergic sensitization *in vivo* [302]. The same experiment was carried out in C57BL/6 mice [302], which showed small numbers of  $\gamma\delta$  T cells from OVA-tolerized mice selectively suppressing Th2-dependent IgE antibody production, without affecting parallel IgG responses. Challenge of these cells *in vitro* also resulted in high levels of IFN- $\gamma$  production. The mechanism of the tolerogenic effect seemed to be either through the inhibition of the expansion of pre-existing OVA-specific Th2 cells or of the development of OVA-specific Th2 cells [21]. Based on these studies, it is possible that DETC may help in either preventing atopic dermatitis or suppressing the propagation of AD-like lesions in the skin through the production of IFN- $\gamma$  and



consequently the suppression of IgE production. The fact that the NOD. $\delta$ -/- mice housed in CNVC of the present study showed the same levels, as opposed to higher levels, of IgE compared to those of the NOD. $\delta$ +/+ mice housed in CNVC does not support the notion that  $\gamma\delta$  T cells contribute to the prevention of AD-like disease through any significant suppression of IgE production.

Though the pathogenesis of AD is not completely known, almost every kind of cell has been implicated as playing a role, including T cells, keratinocytes, mast cells, and Langerhans cells. The roles of mast cells and Langerhans cells, in particular, have been increasingly implicated and elucidated as part of the pathomechanism in AD. Mast cells are known to be effector cells in IgE-mediated immediate hypersensitivity reactions [303]. Moreover, mast cells have been reported to play an important role in eosinophil infiltration into the lung after allergen challenge [304]. In addition, it has been recently documented that histamine, through stimulation of H<sub>2</sub> receptors, suppresses IL-12 production and stimulates the generation of IL-10 [305, 306]. Such an alteration of local cytokine levels could shift the T<sub>H</sub>1/T<sub>H</sub>2 balance toward T<sub>H</sub>2 dominance, thus augmenting eosinophil recruitment and allergic inflammation. More recently, in a study on OVA-induced skin inflammation in mast cell-deficient (*W/W'*) mice, IFN- $\gamma$  mRNA expression was found to be significantly increased in sensitized skin of *W/W'* mice but not in that of wild-type controls. Additionally, total serum IgE levels were significantly increased after OVA epicutaneous sensitization in *W/W'* mice compared to controls [307]. The results suggested that mast cells regulate IFN- $\gamma$  expression in the skin and IgE levels in the circulation. In light of these findings, it can be postulated that damage to or deficiency of mast cells could lead to increased activity of DETC, such as in overexpressing IFN- $\gamma$ . As



IFN- $\gamma$  can damage keratinocytes [307], it is possible that DETC may contribute not only in down-regulation of cutaneous inflammation, but also in damage to local epithelia in some cases.

Among professional antigen-presenting cells (APC), dendritic cells (DC) show the unique capacity to initiate primary immune responses [308]. Immature DC are characterized by their high capacity for antigen uptake and play primary roles in the immune system in peripheral tissues. The prototype of immature DC are Langerhans cells. Human Langerhans cells variably express a trimeric form of the Fc $\epsilon$ RI receptor, containing one  $\alpha$ - and two  $\gamma$ -chains, but lacking the  $\beta$ -chain characteristic for tetrameric Fc $\epsilon$ RI on mast cells and basophils [284, 285, 309]. In contrast to the latter, ample evidence exists that implicates the role of Fc $\epsilon$ RI+ APC in IgE-mediated delayed-type hypersensitivity reactions in atopic diseases. First, patients with AD exhibit enhanced surface expression of Fc $\epsilon$ RI on Langerhans cells [310]. Second, Fc $\epsilon$ RI has been shown to allow Langerhans cells/DC and monocytes to take up, process, and present antigens to T cells more efficiently [282, 311]. Recently, the engagement of the Fc $\epsilon$ RI was shown to stimulate the production of IL-16 in Langerhans cell-like dendritic cells [312]. The functional role of IL-16 in inflammatory processes is only beginning to emerge, but evidence has shown that IL-16 could be involved in autoimmune disorders [313-315], as well as in the pathogenic aspects of allergic asthma, including production of allergen-specific IgE, recruitment of inflammatory cells to the bronchial mucosa, and development of airway hyper-responsiveness [316-318]. Thus, IL-16 released from Langerhans cells after allergen-mediated activation through Fc $\epsilon$ RI may link IgE-driven and cellular inflammatory responses in diseases such as AD [312]. Kraft et al. (2002) have also



recently shown that NF- $\kappa$ B, known to regulate genes essential for inflammatory responses and DC differentiation and function, is activated upon Fc $\epsilon$ RI ligation in primary human monocytes and DC [319]. Their study also showed that NF- $\kappa$ B activation in Langerhans cells isolated from the epidermis was restricted to donors expressing high Fc $\epsilon$ RI amounts. Additionally, Fc $\epsilon$ RI ligation on monocytes and DC led to synthesis and release of TNF- $\alpha$  and monocyte chemoattractant protein-1 (MCP-1), which is decreased by two distinct inhibitors of NF- $\kappa$ B activation. Thus NF- $\kappa$ B activation represents a novel mechanism by which Fc $\epsilon$ RI on monocytes and DC potentially controls inflammatory reactions [319]. As DETC have been shown to secrete several cytokines, including IFN- $\gamma$ , IL-2, IL-3, and IL-4, as well as the potential to express mRNA for IL-1 $\alpha$ , IL-2, IL-3, IL-4, IL-6, IL-7, IFN- $\gamma$ , TNF- $\alpha$ , TNF- $\beta$ , GM-CSF, and MIP-1 $\alpha$  [119], it is obvious that the interplay between DETC, mast cells, and Langerhans cells, as well as other cells of the immune system, must be quite intricate and complex. With the local, non-redundant regulatory role in cutaneous inflammation recently implicated for DETC, it would be interesting to investigate the condition, i.e. number, morphology, cytokine secretion, of DETC in the different mouse models proposed for human AD. Perhaps one would find varying degrees or ways in which DETC are damaged. Ultimately, defining the role of DETC in AD-like disease in mice may lead to understanding the possible defect that lies in the as-yet undefined, functionally equivalent epidermal  $\gamma\delta$  T cells found in human AD patients.



## B. SPONTANEOUS DERMATITIS AND GENETICS

Research into AD over the last 40 years has been dominated by exploration of the cellular and molecular mechanisms. Much of this work has indeed produced breakthroughs in the understanding of this enigmatic disease, such as the discovery of the role of different T-lymphocyte cell subsets, mast cells, Langerhans cells, eosinophils, immunoglobulins, and cytokines. However, much is still unknown particularly regarding the genetic basis for AD, though family aggregation and twin studies provide evidence of a considerable genetic component of this disease [151, 166].

Genetic analyses sought to elucidate the genetic control of the spontaneous dermatitis observed in NOD. $\delta$ –/– mice of the present study. The spontaneous dermatitis that occurs in  $\delta$ –/– mice housed in conventional, non-ventilated cages is strikingly strain-dependent, strongly pointing to a component of heritability in this phenotype [147]. Clinical and histopathologic signs of dermatitis, as well as increased baseline ear thickness, were found in  $\delta$ –/– mice of the NOD background, while C57BL/6. $\delta$ –/– and wild-type mice showed no clinical or histologic evidence of the phenotype. When F<sub>1</sub>, F<sub>2</sub>, and (F<sub>1</sub> X NOD) BC. $\delta$ –/– offspring of NOD. $\delta$ –/– and B6. $\delta$ –/– mice were analyzed, the incidence rates of SpD appeared to be consistent with a single autosomal recessive gene inheritance [147]. Interestingly, a genetic analysis for dermatitis performed on NC/Nga and BALB/cA (BALB) strains demonstrated similar results [320]. As mentioned earlier, Matsuda et al. (1997) discovered that NC/Nga mice reared under non-sterile, conventional conditions developed spontaneous signs and symptoms of dermatitis, while BALB mice under the same housing conditions did not. NC/Nga mice were paired with BALB mice to produce F<sub>1</sub>, F<sub>2</sub>, and (F<sub>1</sub> X NC/Nga) backcross progeny. In the F<sub>1</sub>



generation, no animals expressed dermatitis, with no difference shown between reciprocal matings (NC female X BALB male *vs.* NC male X BALB female). The F<sub>2</sub> generation revealed 89 mice to have dermatitis, while 232 mice did not, showing ~25% dermatitis affected rate. Lastly, in the BC generation, 92 mice displayed dermatitis, while 105 animals did not, showing ~50% affected rate. The authors concluded that these incidence rates were consistent with an autosomal recessive gene [320]. Though original analysis of the genetic data for the NOD. $\delta$ –/– and B6. $\delta$ –/– mice was consistent with a single locus as the major determinant for susceptibility, microsatellite mapping studies based on NOD and B6 SSLPs have since revealed a more complex picture. Three candidate *Nod* recessive chromosomal intervals have recently been identified and are presently under investigation [231].

Genome-wide linkage analyses for susceptibility loci for atopic dermatitis have already been performed in two mouse models. As a logical starting point in the genetic analysis of NOD. $\delta$ –/– and B6. $\delta$ –/– mice, the intervals of susceptibility identified from these previous genome-wide studies were used to assess possible linkage to susceptibility in the NOD. $\delta$ –/– mice as well. In the NC/Nga model [152], the locus of the major determinant was tightly linked to six markers in a locus region (termed *derm1*) on chromosome 9. When genomic DNA from NOD. $\delta$ –/– and B6. $\delta$ –/– mice were analyzed for linkage of SpD susceptibility to the *D9Mit105* locus, which had a position close to the loci described for NC/Nga mice, no linkage was found. In fact,  $\chi^2$  value was essentially equal to 0. Analysis in the NOA model [153] found significant association between ulcerative skin lesions and two markers on chromosome 14. Locus *D14Mit262*, whose position is approximately in the center of the interval of the two markers, was chosen to



be analyzed in the NOD. $\delta$ –/– and B6. $\delta$ –/– mice. Again, no linkage to SpD susceptibility was found, with another  $\chi^2$  value  $\approx 0$ . These results clearly indicate that the loci linked to SpD susceptibility are distinct from those found in the previous two mouse models. The findings of the current investigation mentioned above on the three candidate *Nod* recessive chromosomal intervals will indeed contribute significantly to our understanding of this disease, and potentially to that of human AD. It will be interesting to determine which functional candidate genes are located near the susceptibility regions identified. For example, in the NC/Nga mice, seven candidate genes were found to be near the *derm1* locus, including thymus cell antigen 1  $\theta$  (*Thy1*), CD3 antigen  $\delta$ ,  $\epsilon$ , and  $\gamma$  polypeptide (*Cd3 $\delta,\epsilon,\gamma$* ), IL-10 R $\alpha$  (*Il10r $\alpha$* ), IL-18 (*Il18*), and C-terminal Src kinase (*Csk*), all of which are involved in T-cell functions [152]. In the NOA mouse model, no specific candidate gene was located near the identified regions on chromosome 14 [153]. However, in a subsequent analysis, the authors identified two candidate loci that could modify the gene responsible for the NOA phenotype, one in the middle of chromosome 7, and the other in the telomeric region on chromosome 13 [154]. The gene for IL-16, which plays a crucial role in recruiting CD4+ cells to sites of inflammation during the acute phase of AD [195], is located near the identified locus on chromosome 7. In the vicinity of the identified locus on chromosome 13, there is a murine gene encoding PIK3R1 (phosphoinositide-3-kinase regulatory subunit, polypeptide 1 [p85 $\alpha$ ]). PIK3R1 regulates the signaling events that lie downstream of the Fc $\epsilon$ RI-mediated activation of tyrosine kinases [321], implicating its role in the degranulation of mast cells and basophils, which initiate the allergic response. Additionally, each of the candidate modifier loci identified in the NOA mouse is syntenic to human chromosomal regions



11q13 and 5q13 [322, 323]. These regions are known as consensus areas of linkage to asthma, atopy, or related phenotypes such as serum IgE level [178, 301, 324, 325]. Indeed, the identification of genes underlying shared human and mouse linkages may provide important insights into the etiology of atopic dermatitis. Particularly in the analysis of NOD mice, it will be of interest to investigate any relation of loci identified for SpD susceptibility to those identified for the pathogenesis of the autoimmune diabetic phenotype found in these mice, such as *Idd3*, *Idd10*, *Idd17*, and *Idd18* on mouse chromosome 3 [326].

Apart from the observation of susceptibility of the NOD background to the SpD phenotype, the results from the present study show a distinctly higher level in baseline total serum IgE concentration in NOD mice, regardless of genotype, compared to B6 mice. Curiously, F<sub>1</sub> progeny showed levels similar to those of B6 mice, while (F<sub>1</sub> X NOD) BC animals had IgE levels similar to those of NOD mice. Such results suggest a possible genetic component to the IgE levels seen in these strains, such as an autosomal recessive gene in the NOD background that leads to higher IgE production. Genetic studies, in fact, indicate that different genes regulate the presence of increased levels of serum total IgE and specific IgE. The genetic variance of serum IgE has been reported to constitute about 50% of the phenotypic variance [151, 327]. To date, several research groups have been involved in attempting to isolate the genes responsible for total serum IgE. In 1994, Marsh et al. were the first to report linkage between serum total IgE levels and chromosome 5q in an American Amish population [173]. A study of Dutch families that same year replicated this finding of linkage to chromosome 5q [174]. Since then, additional studies have replicated linkages to chromosome 5, while others could not



[328]. Recently, Liu et al. (2000) found a significant association between a novel IL-13 coding region variant and both high total serum IgE level and presence of atopic dermatitis [329]. Overall, linkages to chromosomes 1, 2, 4, 5, 6, 7, 9, 11, 12, 13, 14, 15, and 16 have all been reported, with replication of significant linkage to chromosomes 5, 11 and 12 [327]. In sum, studies suggest that higher than normal total IgE and specific IgE levels in some atopic individuals are genetically controlled. With the finding that NOD mice, regardless of phenotype, have higher total serum IgE levels, compared to B6 mice, it would be of interest to identify possible genes that may be controlling total IgE levels in NOD mice. Though total IgE does not appear to correlate directly with clinical dermatitis in these mice, the genes controlling their circulating levels may in fact be tightly linked to those conferring susceptibility to SpD.

Notably, results of genetic studies have also suggested genetic linkage of atopic IgE responsiveness (increase in IgE levels) to environmental factors. For example, genetic linkage of atopic IgE response to chromosome 11q13 [330] has been replicated by several groups [297, 299, 331]. The gene for the  $\beta$  chain of the high affinity receptor for IgE (Fc $\epsilon$ RI- $\beta$ ) is a candidate for the chromosome 11q13 effect [177]. FC $\epsilon$ RI- $\beta$  polymorphisms called Leu181/Leu183 have been found to be significantly associated with atopy as well as bronchial hyperresponsiveness [300]. Such genetic influences on IgE responsiveness to environmental triggers, and not just on total serum IgE levels at baseline, may be responsible for the marked differences seen in IgE levels among the different mouse models discussed in the previous section. For example, NOD. $\delta$ -/- mice may be reacting to appropriate environmental allergens as manifested by spontaneous dermatitis, but are not mounting a robust IgE response due to a genetic constraint



compared to NC/Nga mice or DS-Hd mice. Thus, their total IgE levels and associated clinical manifestation of allergic cutaneous inflammation remain of lower severity compared to the other mice.

The study of genetics offers a structured means of investigating complex diseases with a genetic component. A number of advancements can be made through the elucidation of the genetic factors [248]. First, improvements can be made in the definition of disease. For example, various genetic polymorphisms may correlate with specific disease characteristics and may be used to define subgroups of the disease, such as particular responses to particular treatments. Genetic studies will also provide results that will help to define targets of therapeutic intervention and offer a rational means of choosing targets in the pathogenetic pathway of allergic inflammation. Finally, genetic studies can help to define people at risk, leading to rational strategies for the prevention of disease in genetically susceptible children. The understanding of the etiology and heritability of human atopic dermatitis will indeed be accelerated with the use of appropriate mouse models, through which the identification of genes is much more feasible and will help to dissect this incredibly complicated disease.

### **C. $\gamma\delta$ TCR LIGAND**

The overall goal of this project was to develop a Rat Basophil Leukemia (RBL) bioassay in order to test activation through the V $\gamma$ 5/V $\delta$ 1, DETC-specific TCR. Constructs comprised of TCR V $\gamma$ 5 and V $\delta$ 1 chains ligated into pCDL-SR $\alpha$ 296 vector using Xho I and Bgl II sites were developed. The extracellular domain of V $\gamma$ 5 and V $\delta$ 1



were directly fused to the thrombin linker region and CD3 $\zeta$ , which provides the transmembrane domain for both chains. The constructs were successfully transfected into the RBL-2H3 line, which should not express NKG2D, CD8, or other receptors expressed by  $\gamma\delta$  T cells, and theoretically avoids the problem of cell activation through a receptor apart from the isolated transfected  $\gamma\delta$  TCR. Stably transfected cells were demonstrated by positive staining with either GL3 antibody, detecting the C $\delta$  portion of the V $\delta$ 1 chain, or 17D1 MoAb, which recognizes the V $\gamma$ 5 and V $\delta$ 1 chains expressed together. One clone which showed a relatively high percentage of GL3-positive cells (28%) upon initial analysis was subsequently sorted into GL3-positive-only cells and “enriched” into a population eventually showing cells that were 81% GL3-positive and 65% 17D1-positive. These percentages showed a ratio of 17D1 to GL3 positive cells equal to 0.8:1, i.e. 80% of cells staining positively for GL3 were also detected by 17D1. Staining of the RBL tranfectants revealed no interference in the binding of one MoAb by the other. In other words, clone #45 cells exhibited 84% of cells to be GL3-positive when stained only with PE-GL3, and 80% of cells to be GL3-positive when cells were stained with PE-GL3, followed immediately by 17D1 and FITC-conjugated anti-rat IgG + IgM, demonstrating only a negligible difference in percentages of positively-stained cells. Likewise, clone #45 cells before they were “enriched” were analyzed by staining with MoAbs and FACS, also showing no interference in the binding of the MoAbs to the cells. In this case, cells showed 29% to be GL3-positive when stained with PE-GL3 alone, 21% to be 17D1-positive when stained with the 17D1 MoAb alone, and 29% and 23% positive cells for GL3 and 17D1, respectively, when stained with PE-GL3 first, followed immediately by 17D1 and FITC-conjugated anti-rat IgG + IgM. Notably, the



same percentages of positive cells were found when the cells were stained in different order, i.e. 17D1 first, followed by PE-GL3, and then anti-rat IgG + IgM. This observation indicates that there is no preferential binding of one particular antibody over the other to the TCR, which would prevent one from accurately detecting the presence of its target, namely the C $\delta$  or V $\gamma$ 5/V $\delta$ 1 TCR. These findings are consistent with previous results of DETC cell analyses using these MoAbs together, where the same percentages of GL3-positive and 17D1-positive cells are found regardless of staining cells separately or concurrently with the Abs (Tigelaar and Lewis, personal communication). Importantly, analysis of clone #45 cells before they were “enriched” revealed the exact same 17D1 to GL3 positive cell ratio as that found in clone #45 sorted and “enriched” cells, i.e. 23% 17D1-positive of 29% GL3-positive is equal to a 0.79:1 ratio, which is essentially the same as a ratio of 0.8:1.

The percentage of GL3-positive cells was consistently higher than that of 17D1-positive cells. The small discrepancy between these percentages can be explained in two different ways. One is that the V $\delta$ 1 chain, as detected by the staining of C $\delta$  by GL3, is indeed expressed alone in 20% of the RBL transfectants of clone #45, without the necessity of being associated with another chain, namely the V $\gamma$ 5 chain. It is unclear if the same finding of GL3-positive cell predominance would be revealed in other clones as well. Such a trend would indicate that the V $\delta$ 1 chain is either more efficiently transfected into the RBL cells compared to the V $\gamma$ 5 chain, or that the V $\delta$ 1 chain is more stably expressed on the RBL cell surface. The reason for such findings would be unclear. An adjunctive study would include staining the clonal population with F536 MoAb (PharMingen), which is an antibody specific for the V $\gamma$ 5 protein, in order to determine if



the V $\gamma$ 5 chain alone could be expressed. It would make a useful comparison to assess the percentage of F536-positive cells that are also 17D1-positive.

Alternatively, the reason for a higher percentage of GL3-positive cells compared to 17D1-positive cells could be that only 80% of all RBL V $\gamma$ 5/V $\delta$ 1 transfectants, indeed expressing both chains of the TCR, are folded in the proper 3-dimensional conformation to be recognized by the 17D1 MoAb. The two chains of a T-cell receptor fold in an intricate way, in much the same way as those of a Fab fragment of an immunoglobulin with some distinct differences in the way the domains are formed and interact [76, 332]. It is conceivable that the  $\gamma$  and  $\delta$  chains, after transfection into RBL cells, are in fact expressed at the cell surface, but do not always fold together into the proper formation to be recognized by the 17D1 MoAb. The antigen receptor conformation of this TCR is underlined by the finding that epidermal  $\gamma\delta$  cells, similar to most B cells and  $\alpha\beta$  T cells, may be associated more with an antigen receptor conformation than with simple linear epitopes specifically encoded by the V $\gamma$  and V $\delta$  gene segments normally used by DETC [73]. The 17D1 MoAb, thought to react only with cells expressing both V $\delta$ 1 and V $\gamma$ 5, has been found to recognize V $\gamma$ 1/V $\delta$ 1 TCRs in the absence of V $\gamma$ 5+ cells. Therefore, just as the 17D1 MoAb appears to recognize TCRs of a particular conformation regardless of particular gene segments used, it would also fail to recognize TCRs that do not express the proper conformation despite having the V $\gamma$ 5 and V $\delta$ 1 genes. Indeed, it would be advantageous to clarify if the 20% of GL3-positive, 17D1-negative cells are indeed F536-positive, which would imply that both the  $\gamma$  and  $\delta$  chains are being expressed but in a conformation unrecognizable by 17D1. A salient question is whether such a TCR not in the same conformation as that which the majority of DETC share, as detected by 17D1, is



still functional in recognizing the DETC-specific ligand(s), becoming activated, and successfully transmitting signals to the cell in order to carry out DETC-specific activities. Further experiments will indeed be needed to elucidate the requirements for function of the DETC TCR, such as the specific  $\gamma$  and  $\delta$  gene segments used or the folding of the  $\gamma$  and  $\delta$  chains to produce a certain TCR conformation.

Clone #45 “enriched” (81% GL3-positive and 65% 17D1-positive) cells were used in an experimental  $^3\text{H}$  serotonin release bioassay in an attempt to demonstrate that the expressed TCR was able to induce signal transduction through TCR $\zeta$ , and thus to activate the transfectants. This first assay showed mixed results. First, the experimental conditions of incubation with GL3 hybridomas and 17D1 soluble antibody failed to induce serotonin secretion by the RBL transfectants, implying a lack of TCR activation. Both GL3 hybridomas and 17D1 soluble antibody were designed to act as positive controls, assuming that these antibodies would bind the  $\text{V}\gamma 5/\text{V}\delta 1$  TCR and induce activation. Other studies (Steele, unpublished data) have shown the effective stimulation and release of  $^3\text{H}$  serotonin-loaded  $\text{V}\gamma 7/\text{V}\delta 5$  RBL transfectants by the GL3 hybridoma in a dose-dependent fashion. A ratio of 10:1 of GL3 hybridomas to RBL cells was found to induce up to 20% serotonin release (Steele, unpublished data). Although a 10:1 ratio of GL3 hybridomas to RBL cells was also used in the assay of the present study, no serotonin release was induced. Because GL3 MoAb detects the C $\delta$  chain, with no constraining need for recognition of a particular conformation, it is expected that the GL3 hybridomas in sufficient concentration should stimulate the RBL  $\gamma\delta$  transfectants. Future assays can incorporate varying ratios of GL3 hybridomas to RBL cells and to determine if there is a dose-dependent effect. It is conceivable that the manner in which the TCR is



expressed on the RBL transfector causes the GL3 hybridoma to bind inefficiently to the TCR, perhaps enough to be detected as seen in FACS analysis, but does not cross-link the TCR strongly enough to stimulate activation and release. A higher GL3 hybridoma to RBL cell ratio may result in functional activation through the TCR. GL3 may also have varying binding affinities to different specific receptors, perhaps being lower for V $\gamma$ 5/V $\delta$ 1 in comparison to V $\gamma$ 7/V $\delta$ 5 (a receptor characterizing murine intestinal cells) [333]. In future assays, GL3 free antibody, as well as bead-bound GL3, such as to PGS beads, can be used to provide a direct comparison between intercellular engagement of ligand with TCR and that of soluble antibody with the TCR. It may be found that the assay is more suitable for detection of activation via one particular method over another.

Finally, it is possible that the physical constraints of GL3 antibody surface expression on the hybridoma cell are preventing efficient cell-cell interaction and antibody-TCR engagement. In theory, F(ab')<sub>2</sub> fragments of the GL3 antibody could be used to assess activation through the TCR. The F(ab')<sub>2</sub> fragment is produced by the digestion action of the protease pepsin on the carboxy-terminal side of the disulfide bonds of the immunoglobulin. The two antigen-binding arms of the antibody molecule remain linked, retaining the same antigen-binding characteristics as the original antibody [2]. Using such fragments of the antibody to activate cell-surface TCR would bypass the potential physical constraints of antibodies expressed on the surface of another cell.

Clone #45 RBL transfectors also did not show stimulation by 17D1 MoAb. Several different explanations can account for this finding. One, the concentration of 17D1 MoAb in the unpurified supernatant collected from cultures of 17D1 hybridoma cells is unknown (Lewis, personal communication). In this study, 50  $\mu$ l of the



supernatant was added directly to the well containing the Clone #45 cells. It is conceivable that the concentration of 17D1 MoAb in the supernatant is in fact rather low such that 50  $\mu$ l alone did not contain adequate amounts of antibody to activate the TCR on the RBL transfectants. In future assays, both higher volumes or concentrations of 17D1 MoAb in the supernatant can be used to assess activation via this antibody. Logically, use of purified supernatant, containing a concentrated amount of 17D1 MoAb alone, would have a much higher likelihood of demonstrating TCR activation. An alternative explanation for the lack of transfectant cell stimulation by 17D1 is that the MoAb could not induce detectable activation via the TCR on the RBL cells. It is possible that the V $\gamma$ 5/V $\delta$ 1 TCR is expressed on the RBL cell surface in such a way that 17D1 MoAb does not bind accurately enough to induce activation through CD3 $\zeta$ , i.e. does not effectively cross-link the TCR. Additionally, staining of the RBL transfectants with FITC-conjugated anti-17D1 showed 65% of the “enriched” Clone #45 cells to be 17D1-positive. This percentage is lower than the ideal percentage of >99% purity of 17D1-positive cells in a clone. Any of these factors may have contributed to the finding of failure of activation by 17D1 MoAb, or activation at a level below detectable using this assay system.

Though the RBL  $\gamma$  $\delta$  transfectants failed to show activation by the GL3 hybridomas and 17D1 soluble MoAb, they did demonstrate serotonin release in response to PDV keratinocytes. This result is intriguing. One possible interpretation is that the V $\gamma$ 5/V $\delta$ 1 TCR was in fact activated by the keratinocytes. DETC have been proposed to recognize a unique set of molecules that may be expressed by “stressed” neighboring cells [33], such as keratinocytes in the epidermis with which DETC are in intimate



contact. In fact, Havran et al. (1991) demonstrated that DETC may be stimulated to produce lymphokines by interaction with transformed keratinocytes in vitro [105]. The stimulation was mediated through the DETC receptor and did not appear to be subject to MHC restriction. This finding provides support for the hypothesis that DETC can recognize self-antigens and may participate in immune surveillance for cellular damage rather than for foreign antigens [105]. In addition, this proposed functional role of DETC is consistent with the extraordinary homogeneity in the TCR  $\gamma$  and  $\delta$  gene utilization, which implies a very limited repertoire for the antigen(s) recognized by the receptor [42]. Thus, it is possible that the TCR on the RBL transfectants successfully engaged with ligand on the keratinocytes, induced activation through CD3 $\zeta$ , and stimulated serotonin release. The nature of the ligand is unclear and cannot be elucidated from this assay. The keratinocytes used in this assay were neither heat-shocked nor infected, or otherwise manipulated to cause purposeful damage. Yet, they had been removed from culture in a flask by scraping just prior to incubation in the assay with the RBL transfectants. Therefore, it is conceivable that this kind of physical “stress” and manipulation caused expression of a molecule which was recognized by the DETC TCR.

An alternative explanation is that the RBL transfectants were stimulated to release serotonin through a mechanism not related to TCR activation. Several different stimuli have been found to transduce cell activation signals in DETC. Such signals include: a) mitogenic lectins [58, 334]; 2) coculturing with a transformed keratinocyte line, Pam 212 [105], as described above; 3) epicutaneous application of irritant chemicals [110]; and 4) epidermal injury caused by injected autoreactive cytotoxic T-cells [335]. The mechanism through which these cell activation signals act on the DETC is not clear. Activation leads



to production of IL-2, the expression of IL-2 receptor, and vigorous proliferation [58]. It is hypothesized that the same signals also lead to the “maturation” of DETC that then exhibit “killer leukocyte” activity towards tumors or otherwise transformed cells. IL-7 produced locally by keratinocytes has been found to promote the growth of DETC in vitro [112, 336]. Thus, IL-7 from keratinocytes may also contribute to this maturational process of DETC into “killer cells” [109]. It is possible that the RBL  $\gamma\delta$  TCR transfectants are receiving as-yet unidentified cell activation signals from the keratinocytes through a non-TCR-related mechanism and are likewise transformed into a cell of “increased” activity. Such activity could lead to serotonin release.

It is also conceivable that the RBL  $\gamma\delta$  transfectants were activated through an alternative receptor molecule aside from the V $\gamma$ 5/V $\delta$ 1 TCR. For example, NKG2D is a receptor for the distant MHC class I homologue called MICA [98]. NKG2D has recently been detected on most  $\gamma\delta$  T cells, as well as on CD8+  $\alpha\beta$  T cells and NK cells. Engagement of NKG2D can activate cytolytic responses by  $\gamma\delta$  T cells and NK cells against epithelial tumor cells and transfectants expressing MICA [98]. Interestingly, the NKG2D ligands of the MHC class I-like molecules called Rae-1 and H60 have been found on skin cells exposed to carcinogens [100]. It is not definitively known whether RBL cells possess the NKG2D receptor. It is possible that Rae-1 and H60-like molecules are being expressed on the keratinocytes, which are recognized by NKG2D or other as-yet unidentified receptor on the RBL cell, thus causing activation and release of serotonin through a non-TCR-dependent mechanism. Recently, a monoclonal antibody specific for murine NKG2D has been generated [337]. This anti-NKG2D MoAb could be used to stain and analyze the RBL cells for detection of this receptor. One could also use the



MoAb as a testing condition in the RBL bioassay, in which serotonin release may indicate the presence of this receptor on the RBL surface. Additionally, RBL  $\gamma\delta$  transfectants pre-blocked with NKG2D MoAb could be incubated with the keratinocytes to assess any difference in serotonin release. Failure of RBL cells to release serotonin in this condition would highly suggest the presence of NKG2D receptor.

Several steps can be taken to optimize the RBL bioassay. One of the first steps would be to further “enrich” the Clone #45 cell line into an ideally pure, >99% 17D1-positive clone. Though it is not certain whether GL3-positive cells do in fact possess both the  $\gamma$  and  $\delta$  chains and are not being detected by 17D1 due to conformational differences, such cells would ideally not be included in the purified clone until such clarification is made. Testing conditions with GL3 hybridomas and 17D1 soluble MoAb can be adjusted as discussed above to optimize the sensitivity of the bioassay. A suitable target to effector cell ratio of possible ligand-bearing cells and transfectants would need to be determined. An alternative method is to use homogenates of potential ligand-bearing cells, in which the proteins in the homogenates could be used to attempt activation of RBL cells in the bioassay. Finally, in order to definitively demonstrate that activation is indeed occurring through engagement of a particular ligand with the TCR, production of soluble  $V\gamma 5/V\delta 1$  TCR (sTCR) will play a key role [338]. Once a particular type of cell is shown to activate the RBL  $V\gamma 5/V\delta 1$  transfectant, sTCR can be used to attempt blocking of activation. If such blocking is achieved, characterization of the activating molecule on the ligand-bearing cell can begin. Ultimately, this RBL bioassay with TCR transfectants has the exciting potential to help identify the ligand(s) for the



strikingly homogeneous V $\gamma$ 5/V $\delta$ 1 TCR-bearing dendritic epidermal T cell. Surely, the elucidation of such ligand(s) will radically increase the understanding of the physiologic and functional roles of this intriguing subset of epidermis-restricted T cells.



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